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R. MANNINGER, I. NÁSZ, K. RAUSS, F. B. STRAUB, J. SZIRMAI,
K. VASS, J. WEISSFEILER

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TOMUS XV

FASCICULUS 1



AKADÉMIAI KIADÓ, BUDAPEST

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SIGNIFICANCE OF LYMPHOPENIA IN THE PATHOGENESIS OF WASTING SYNDROME IN NEONATALLY THYMECTOMIZED MICE

By

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(Received June 21, 1967)

Summary. The response to cold stress of neonatally thymectomized mice differed from the response of normal animals. The thymectomized mice exhibited an increased susceptibility to cold and a decreased lymphopenic reaction after the exposure. It has been concluded that the thymus plays a primary role not only in immunological but also in general adaptation.

Mice thymectomized in the neonatal period develop a general immunological areactivity and a special condition the so-called wasting syndrome [1, 2].

After an accidental fall in the temperature of the room where neonatally thymectomized mice were kept, massed death of the animals was observed. Normal animals of the same litters withstood the low temperature without injury. This observation indicated that thymectomized mice develop not only immunological but also other adaptational disturbances. Since the phenomenon has so far not been described, it seemed interesting to examine the response to experimental cold stress of mice with wasting syndrome. As the absolute lymphocyte count in such animals remains at low levels throughout life, it was examined whether the lymphopenic reaction characteristic of normal animals exposed to cold occurred in the same way in thymectomized mice.

Materials and methods

Animals. Inbred 6 to 8 week old mice of strain C₃H were used. The experiments were carried out on 20 animals which had been thymectomized 24 hours after delivery as described by MILLER [3]. The same number of normal mice of the same litters served for controls.

Cold stress and lymphopenic reaction. The animals were exposed to 4° C ambient temperature for 4 hours. Blood samples for lymphocyte counts were taken from the orbit before the experiment. Four hours after cooling the survivors were sampled in a similar manner.

Development of wasting syndrome was estimated from the loss of body weight and low absolute lymphocyte counts.

Results

Within 2 hours after exposure to cold 3 out of the 20 thymectomized mice died. In the test group the severest signs of wasting syndrome had developed in these very animals (lowest weight and lymphocyte count, dermatitis, diarrhoea). The data are shown in Table I.

Table I

Data for neonatally thymectomized mice dying in 2 hours after exposure to cold

Designation of animal	46	47	49
Body weight, g*.....	9	9	7
Dermatitis and diarrhoea	+	+	+
Absolute lymphocyte count**	400	560	480

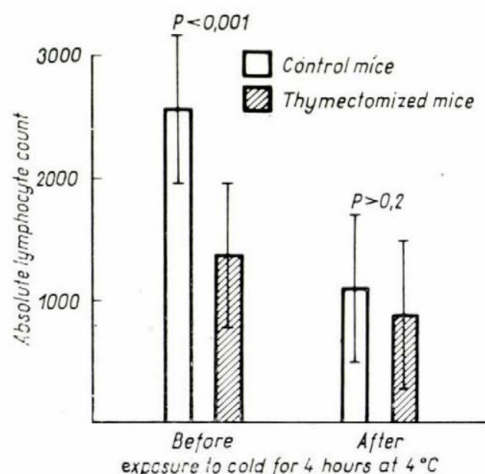
* Weight of thymectomized mice: 12.3 ± 2.8 g.** Absolute lymphocyte count in thymectomized mice: 1400 ± 800 .

Fig. 1. Effect of exposure to cold on absolute lymphocyte count in neonatally thymectomized and control mice

Four hours after cooling all control animals showed the usual lymphopenic reaction: the absolute lymphocyte count decreased on the average from 2600 to 1100. The lymphopenic reaction was considerably weaker in thymectomized mice; the average lymphocyte count decreased from 1400 to 900. Thus, the decrease in the control group was 60%, in the test group, 35%. The average absolute lymphocyte count was in both groups in the range of 1000 (Fig. 1).

Discussion

Our accidental observation and experimental data indicate that animals with the wasting syndrome are not only lacking specific immunological adaptation but their general adaptation capacity is also impaired. Cold stress was fatal to 3 out of 20 thymectomized mice, while the control animals withstood the same effect. The 3 dead mice had shown the severest wasting syndrome observed in the test group. The lymphopenic reaction was significantly less definite in thymectomized mice surviving the cold stress than in the control group.

No data have been available as to the effect on general adaptation of neonatal thymectomy. There are, however, data for the physiological reaction of the thymus in general adaptation processes. In the alarm reaction, the thymus loses considerable weight and the circulating lymphocytes disintegrate [4—6]. The immunological reaction to the antigenic stimulus is simultaneous with the losing weight of the thymus [7, 8]. The alteration of the thymus associated with both the alarm reaction and the development of immunity may be due to the outflow of lymphocytes. Thymectomized animals are unable to respond according to SELYE's principle to stimuli eliciting stress.

Our experiments indicate that animals thymectomized after birth lose their ability to react with lymphopenia to exposure to cold. In view of this finding the following data should be considered.

(1) The absolute lymphocyte count decreased to the same level (about 1000) after cold stress in both the test and the control groups.

(2) Thymectomized animals with lymphocyte counts higher than 1000 survived the exposure to cold. These animals, although in a lower degree, were able to produce the lymphopenic reaction.

(3) The absolute lymphocyte count in mice succumbing to the cold stress was lower before the exposure than that in control animals after the stress.

It has been described that the lymphocyte count in mice thymectomized after birth remains at a low level for their life and this condition is associated with the immunological areactivity of such animals [9]. On the basis of our observations it may be assumed that thymectomized animals fail to produce those lymphocytes which are responsible for the lymphopenic reaction developing after stress and, in addition to immunological areactivity, the absence of these lymphocytes is responsible for the other disturbances affecting general adaptation.

Summarizing the problem, it seems probable that, although the primary role of the thymus has been confirmed only in immunological adaptation, this and other organs performing similar functions play a primary part in general adaptation.

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EFFECT OF HYPERBARIC OXYGENATION ON THE LOCAL SHWARTZMAN PHENOMENON

By

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(Received July 12, 1967)

Summary. As compared to control animals kept in normal air atmosphere, in rabbits placed in 3 atm pure oxygen the Schwartzman phenomenon developed more rapidly and with higher intensity. Hyperoxia itself induced no Schwartzman phenomenon, but enhanced the effect of the eliciting endotoxin dose. Hyperbaric oxygenation exerted no effect on the Thomas reaction (adrenalin + endotoxin). Hyperoxia is assumed to enhance the Schwartzman phenomenon by impairing the lysosomal membrane.

A number of recent observations indicate that granulocyte lysosomes play an important role in the pathogenesis of inflammation, haemorrhage and necrosis developing in the local Schwartzman reaction. THOMAS [1] was able to induce Schwartzman phenomenon with a preparatory dose of leukocyte granules and with an eliciting injection of endotoxin. Endotoxin is one of the most effective factors impairing the lysosomal membrane [2, 3]; it causes the disruption of the membrane and a subsequent release into the intra- and extra-cellular area of enzymes decomposing protein, nucleic acid and polysaccharides. These hydrolases are responsible for the lysis of the cell. HALPERN [4] inhibited the local Schwartzman phenomenon with non-specific protease inhibitor. SZILÁGYI et al. [5] found that, probably due to the decrease of enzyme activity, the phenomenon was inhibited by hypothermia.

SLEDGE and DINGLE [6] and ALLISON [7] have shown that the lysosomal membrane was considerably damaged by hyperoxia. In view of this finding the effect of hyperbaric oxygenation on the local Schwartzman phenomenon has been studied.

Materials and methods

Local Schwartzman phenomenon was induced by the semiquantitative method of KESZTYÜS *et al.* [8] on the dorsal skin of rabbits. The preparatory injections consisted of different doses of *E. coli* O111 endotoxin. The challenging dose was administered 24 hours later intravenously. The control animals were kept in normal laboratory atmosphere. Hyperbaric oxygenation was performed in the chamber presented in Fig. 1. The chamber, 20 litres in volume, was suitable for the reception of 2 rabbits. CO₂ and moisture were removed with NaOH and CaCl₂, respectively, and pure oxygen was flowed through the chamber during the experiment. Pressure was raised to 3 atm in 30 minutes and maintained at this level for 60 minutes. Reduction of the pressure lasted for about 30 minutes. The temperature and humidity of the chamber atmosphere was practically constant throughout the experiment.

THOMAS' reaction was induced by injecting at the same time 300 µg adrenalin intracutaneously and endotoxin intravenously. The results were read after 24 hours.

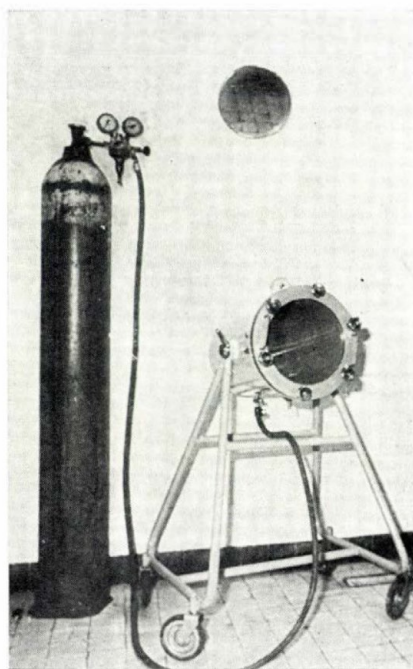


Fig. 1. Hyperbaric chamber

Results

It is well-known that the maximum intensity of the Shwartzman phenomenon develops about 24 hours after the challenging injection. Two hours after the eliciting injection the control animals kept in normal air atmosphere showed but traces of the reaction. In contrast, the phenomenon developed very rapidly in rabbits placed in the hyperbaric chamber (Fig. 2). The 24 hour results are summarized in Fig. 3. It is evident that after full development the intensity of the Shwartzman reaction was considerably higher in hyperoxygenated than in normal animals. It is also remarkable that, as seen from the comparison of data in Fig. 2 and Fig. 3, the hyperoxygenated group developed a practically complete Shwartzman reaction as soon as 2 hours after challenging.

In subsequent experiments we examined the degree of oxygenation needed for increasing the Shwartzman reaction. Under the above experimental conditions about 2 atm 100% oxygen was necessary. It was also shown that hyperoxia (3 atm 100% oxygen) exerted no effect on the skin reaction induced with adrenalin + endotoxin [9].

When the animals were placed in 3 atm air, development and intensity of the Shwartzman phenomenon were similar to those observed in rabbits

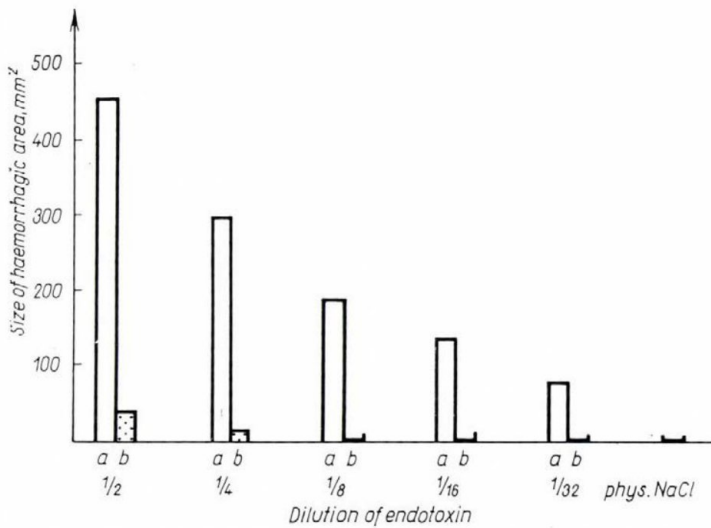


Fig. 2. Shwartzman reaction 2 hours after challenging. Averages of 15 experiments.
a = 3 atm 100% oxygen; b = 1 atm air

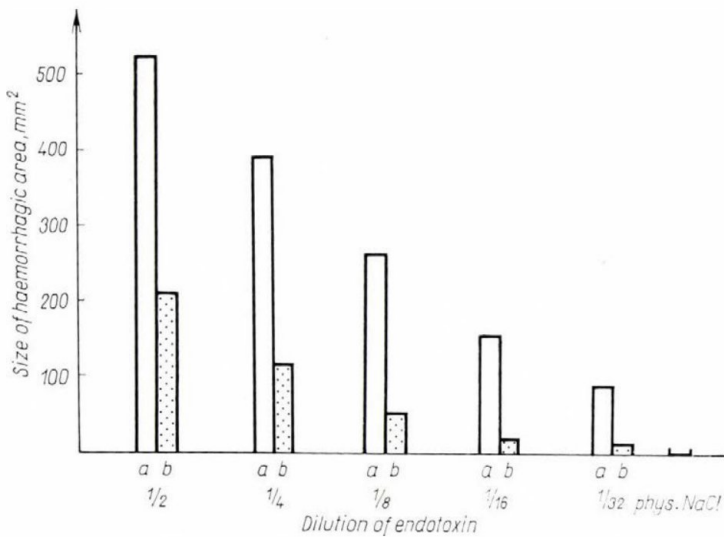


Fig. 3. Shwartzman reaction 24 hours after challenging. Averages of 15 experiments
a = 3 atm 100% oxygen; b = 1 atm air

kept in normal air atmosphere (Fig. 4). The Shwartzman reaction failed to develop in 3 atm pure oxygen when the rabbits had been given only preparatory but no eliciting doses.

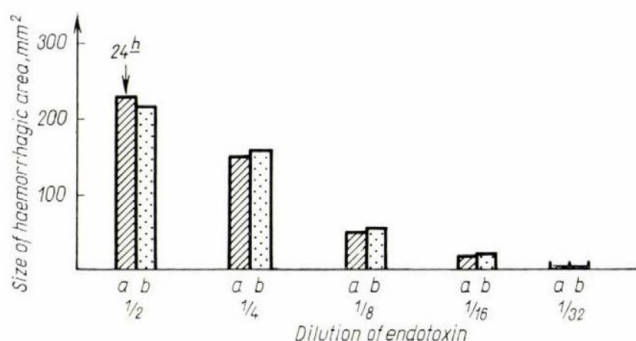


Fig. 4. Schwartzman reaction 24 hours after challenging. Averages of experiments.
a = 3 atm air; b = 1 atm air

Discussion

The present experiments have clearly shown that hyperoxia highly enhances the development and considerably increases the intensity of the Schwartzman phenomenon. An increase in tension *per se* does not influence the reaction. Hyperoxia of the tissues may act on haemorrhagic and necrotic processes partly by impairing the lysosomal membrane and thus releasing enzymes, partly by oxidating the SH groups of cathepsin-type enzymes into SS-bonds. In the Schwartzman phenomenon the main factor is the damage of the membrane. This occurs promptly and leads to a very rapid development of the phenomenon. The increase in the intensity of the reaction is probably due to the effect of hyperoxia increasing the tissue damage caused by the endotoxin. On the other hand, inactivation of the released enzymes does not seem to influence the development of Schwartzman phenomenon. It has been shown that without the eliciting dose hyperoxia is unable to start the above mechanism. Thus hyperbaric oxygenation exerts an adjuvant effect. The observed effect of hyperoxia is another indirect proof of the important role of lysosomes in Schwartzman phenomenon.

Recent observations indicate that blood clotting disturbances may contribute to the development of the Schwartzman phenomenon [10]. Thus hyperoxia is supposed to act in the same manner. The effect of either hypoxia or hyperoxia on blood clotting has not been fully elucidated [11, 12]. It cannot be excluded therefore that hyperbaric oxygenation may influence the Schwartzman phenomenon by this way.

The fact that hyperbaric oxygenation failed to affect the Thomas reaction is indicative of a difference between the said reaction and Schwartzman phenomenon.

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CULTIVATION OF TOXOPLASMA GONDII IN PRIMARY HUMAN AMNION CELL CULTURE

By

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(Received July 22, 1967)

Summary. The susceptibility to *T. gondii* of primary human amnion and HeLa cell cultures has been compared. The degree of multiplication was estimated from the number of intracellular Toxoplasma. Human amnion cells were highly superior to HeLa cells for culturing the parasite.

Cultivation of Toxoplasma by means of methods less expensive and less dependent on external factors than animal experiments has long been the goal of several investigators. Toxoplasma being an obligate intracellular parasite, studies have been performed on the applicability of tissue cultures used in virology [1–6]. In recent years several authors have described methods for culturing and even maintenance of the organism in various tissue cultures [7–9].

The present experiments have been based on the consideration that the primary human amnion cell culture consists of epithelial cells close to the embryonic stage and it is well-known that Toxoplasma has a high affinity to embryonic tissues.

Material and methods

Organism. *T. gondii* type strain RH was used. The strain was maintained by weekly passages in mice. Three days after infection the animals were sacrificed by breaking the cervical vertebrae and then the abdominal cavity was washed with 4 ml saline ("abdominal exudate"). Before inoculating into tissue cultures the fluid was tested for bacteriological sterility. Two kinds of culture were used: (1) 0.1 ml aliquots of the abdominal exudate were transferred into tissue culture tubes in which the nutrient medium had been changed on the previous day; (2) 0.1 ml aliquots of the abdominal exudate and of its 1 : 10 and 1 : 100 dilutions were inoculated into tissue cultures.

Tissue cultures. Primary human amnion tissue culture was prepared by trypsinization. Hanks' solution containing 10% human serum and 0.4% lactalbumin hydrolysate was used as a maintenance medium. The medium was changed at 5 or 6 day intervals. The parasite was inoculated into 8, 10, 18, 24, 30 and 41 days old tissue cultures.

The HeLa cell line has been maintained in our laboratory for years. The medium consisted of Hanks' solution supplemented with 10% rabbit serum and 0.4% lactalbumin hydrolysate. Before inoculation the rabbit serum medium was replaced by human serum medium.

In some experiments trypsinized HeLa cells were added to the developed human amnion cell monolayer. In such cultures HeLa cells formed islets which could well be distinguished from amnion cells in both unstained and stained preparations. The media were supplied with the usual antibiotics. In each test tube a 7 × 15 mm glass plate cleaned and sterilized in a similar manner as the tubes was placed. The amnion, HeLa and mixed tissue cultures were infected simultaneously with one sample of abdominal exudate taken within 2 days before inoculation.

The plates were removed from the tubes, fixed in methanol, stained with Giemsa and examined under low and high power. The result was compared with the microscopic picture of the corresponding unstained culture.

Results

Amnion cells after 48 hours were shown to be infected in 80 to 90%. The cytoplasm contained up to 18 rosettes (Fig. 1). By the 72nd hour, most

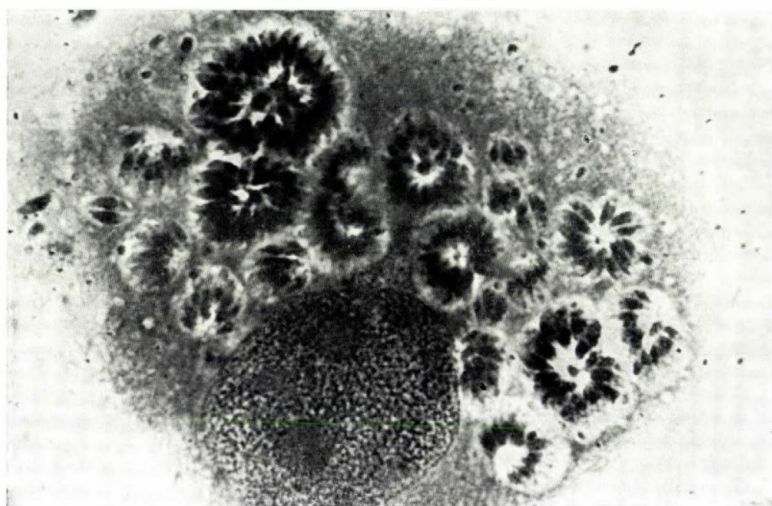


Fig. 1. *Toxoplasma* arranged in rosettes are present in the amnion cell 48 hours after infection

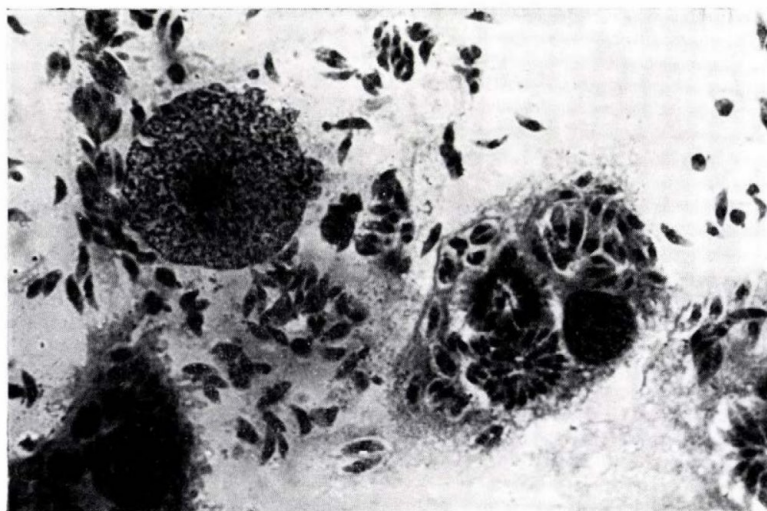


Fig. 2. A few hours later some rosettes are still in the cells, but most of them are found extracellularly

cells had disintegrated and the organisms were arranged extracellularly around the nuclei of destroyed cells (Figs. 2 and 3). Non-infected cells, except for a small degree of vacuolization, retained their normal structure and staining.

On the basis of their staining property, two kinds of HeLa cell were distinguished. The majority had a bright blue cytoplasm and a dark blue nucleus. Cells belonging to this type contained the parasite but occasionally.

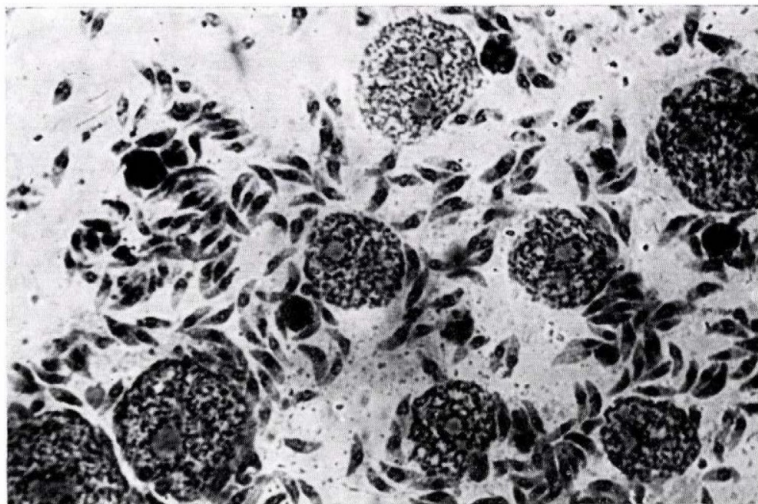


Fig. 3. After 72 hours all infected amnion cells are destroyed. The released parasites are arranged in the neighbourhood of the nuclei



Fig. 4. Some HeLa cells show the evidence of *Toxoplasma* multiplication. Non-infected cells are characterized by vacuolization in the cytoplasm

Many cells of the other type characterized by a relatively large faint blue cytoplasm and reddish nucleus contained 1 to 3 rosettes (Figs. 4 and 5). Non-

infected cells were highly vacuolized and the tissue culture had detached from the tube by the third day.

The difference between amnion and HeLa cells in the degree of multiplication of the parasite was clearly demonstrated on mixed tissue cultures. The parasite multiplied well in nearly all amnion cells. In the same microscopic field HeLa cells contained *Toxoplasma* but occasionally (Fig. 6).



Fig. 5. In the advanced stage of infection the parasites are released from HeLa cells

Discussion

In recent years *Toxoplasma* has been cultivated with success in tissue cultures [10, 11]. HeLa cells have extensively been used for this purpose [12–15] as they can be maintained almost indefinitely. In our experiments HeLa cells were compared with primary human amnion cells for susceptibility to *Toxoplasma*. Amnion cells can be maintained for more than one month if the maintenance medium is changed at regular intervals. It has been found that these cells are highly susceptible to *Toxoplasma* independently from the age of the tissue culture: 41 day old cultures were just as susceptible as 7 day old ones. When the tissue culture was infected with undiluted abdominal exudate, 80 to 90% of the cells became infected with the organism in 48 hours. When prior to inoculation the exudate had been diluted 1 : 10, the parasite was present after 72 hours in a similar percentage of the cells. With dilution 1 : 100 multiplication was less intensive; after 72 hours only about 30% of the cells contained rosettes. In mixed tissue cultures, due to the intensive staining of the majority of the cells, the parasite could not be detected reliably in HeLa cells. In contrast, the parasite was excellently visible before the faint grey or light blue background exhibited by the cytoplasm of amnion cells.

Our results indicate that the primary human amnion cell culture is useful for the production of large amounts of *Toxoplasma* needed in serological and biochemical studies. In view of its high susceptibility, the amnion tissue culture is probably also suitable for the isolation of the parasite. The easy detectability of the organism in such cultures is another advantage.

Acknowledgement. The authors are indebted to DR. N. ZOLTAI and DR. M. JANKÓ (National Institute of Public Health, Budapest) for supplying *T. gondii* type strain RH.



Fig. 6. Mixed tissue culture. Intensive multiplication of *Toxoplasma* in amnion cells. HeLa cells show vacuolization

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STUDIES ON SUCROSE BREAKDOWN BY INVERTASE-PRODUCING CLAVICEPS STRAINS

By

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Summary. The β -h-fructofuranosidases of the three invertase-producing *Claviceps* strains were capable of transferring fructosyl units and to synthesize oligosaccharides accordingly in the course of sucrose breakdown. Number, amount and production order of the transfer oligosaccharides varied with each strain. Orientation experiments showed that the main component of the transfer oligosaccharides was fructose, and two components were reducing compounds.

The importance of transfer oligosaccharides produced in sucrose breakdown is discussed in view of the metabolism of the living mycelium.

The carbohydrates and especially sucrose play an important role in the metabolism of alkaloid-producing *Claviceps* strains. Maintenance of cultures, preparation of inocula and fermentations are performed mostly in media containing sucrose [1—12]. It was shown in a previous paper [13] that 3 out of 4 *Claviceps* strains hydrolyzed sucrose extracellularly by invertase; the remaining strain produced an endosaccharase. It was also shown that oligosaccharide-type substances appeared in the medium during sucrose breakdown by invertase positive strains. In view of these findings it was thought advisable to perform a more thorough investigation into this process. The rate of production of these transfer oligosaccharides produced during fermentation as a result of invertase activity [28—30] seems to be important in view of the whole fermentation process, as these products were presumably less effectively attacked by the microorganism. Therefore studies on the possibility of their utilization were also of interest.

Materials and methods

Organisms. Three invertase-producing strains were used; *Claviceps* sp. mutant Ix 1326, *Claviceps* sp. strain T 20 and *Claviceps* sp. strain Se 1. Properties, maintenance and pre-cultivation of the strains have been described previously [13].

Preparation of dried mycelium. Four days glass fermentor cultures were washed three times in saline by centrifugation. The harvest was mixed with 20 volumes of 4°C acetone and filtered through a Büchner funnel. After repeating this procedure the mycelium was left to dry at room temperature then was ground. Before each experiment the ground mycelium was mixed with distilled water so as to give a 20% suspension.

Incubation system. At zero time the above suspension was mixed with an equal volume of a medium containing 1 M sucrose in M/7.5 pH 5.5 phosphate buffer. The system was incubated on a horizontal shaker at 25°C. When the incubation lasted for more than 10 hours, in order to prevent bacterial contamination, 500 µg/ml of chloramphenicol was added. Prelim-

inary experiments showed that at that concentration the antibiotic did not influence the results. Sampling was performed by removing 0.5 ml aliquots at 0, 2, 4, 6, 10, 12 and 20 hour periods. The samples were inactivated for 2 minutes in boiling water. The supernatants obtained after centrifugation were used in the analysis.

For experiments with living cells samples were taken daily from the glass fermentor culture used for the preparation of the acetonized mycelium. The concentration of sucrose in the fermentation liquor was the same as that used in experiments with dried mycelium (0.5 M).

Analytical methods. Paper chromatography was performed on Macherey-Nagel No. 214 paper treated as described by MATTHIAS [14]. Butanol : glacial acetic acid : water (4 : 1 : 1) was used as a solvent. 5 μ l samples were run 3 times repeatedly by the ascending technique. The spots were detected with benzidine-trichloroacetic acid [15], anthrone [16], alkaline silver nitrate [17] and TTC [18].

Total reducing capacity was determined by the method of SOMOGYI [19] and NELSON [20] and expressed in glucose equivalents.

Quantitative determination of glucose, fructose and sucrose was performed as follows. Six parallel chromatograms were run for each sugar. One of the chromatograms for each sugar was developed so as to serve as a guide: from the remaining 5 chromatograms the spots corresponding to the sugar under examination were cut out, sheared to small strips and eluted by shaking in 10 ml distilled water at 60 to 70°C. The eluate was passed through a glass filter, then the filtrate was examined for reducing sugars (glucose and fructose) by the SOMOGYI-NELSON method. Sucrose was determined by the anthrone method.

Preparation of partially purified transfer oligosaccharides. A sample of 100 ml sucrose solution, which had been incubated with acetonized mycelium for 10 hours, was boiled for 2 minutes then poured onto a 30 $\times$ 4 cm column of Celite and activated charcoal (1 : 1 w/w) [22]. Washing was performed with 50 ml aliquots of water until the effluent gave negative SOMOGYI-NELSON test (absence of fructose and glucose), thereafter with 2 per cent ethanol until the effluent gave negative anthrone test (absence of sucrose). Finally the transfer oligosaccharides were eluted with absolute ethanol. The fractions were checked by paper chromatography as described above.

Breakdown of transfer oligosaccharides by yeast invertase. The alcoholic eluate containing the transfer oligosaccharides was concentrated *in vacuo* then taken up in water and treated with BDH invertase and with acetonized suspensions of the following invertase producing yeasts: *Saccharomyces cerevisiae* (baker's yeast strain), *Candida beerwijckii* strain OKI* 478/1961 and *Candida pseudotropicalis* strain OKI* 371/1964 [23–25]. The acetonized organisms were prepared in a similar manner as acetonized *Claviceps* cultures except that the yeasts were cultured in Roux flasks on CSILLAG's molasses agar [26] for 4 days. The BDH invertase (yeast product) was dissolved to make a 0.2 mg/ml solution (0.063 mg protein/ml). The invertase solutions obtained in this manner were mixed at 1 volume proportions with the oligosaccharide solutions and incubated at 25°C. Samplings were performed at 2 hour intervals. The samples were chromatographed as described above. The endoglucosaccharase producing *Candida requiyii* strain OKI XV/1961 [27] was treated similarly except that a cell-free extract of cells disintegrated with quartz sand was used instead of acetonized cells; the extract was incubated at pH 5.9 [28].

Acid hydrolysis of transfer oligosaccharides. The alcoholic eluate obtained from column chromatography was evaporated to dryness in a water bath. The residue was taken up in distilled water and treated in a closed system with 2.5 M sulphuric acid at 100°C overnight. The material was then neutralized and chromatographed as described above.

Results

Examination of sucrose breakdown by the acetonized mycelium of the 3 *Claviceps* strains supplied the following results. When strain Ix 1326 was incubated in sucrose the gradual appearance of 4 carbohydrate spots was observed. Each of these was characterized by an R_f value lower than that of sucrose. Glucose was present at gradually increasing concentrations. Fructose was detected but in traces and during the last third of the incubation period. The 4 new carbohydrates, designated in the order of decreasing R_f values as

* OKI = National Institute of Public Health, Budapest

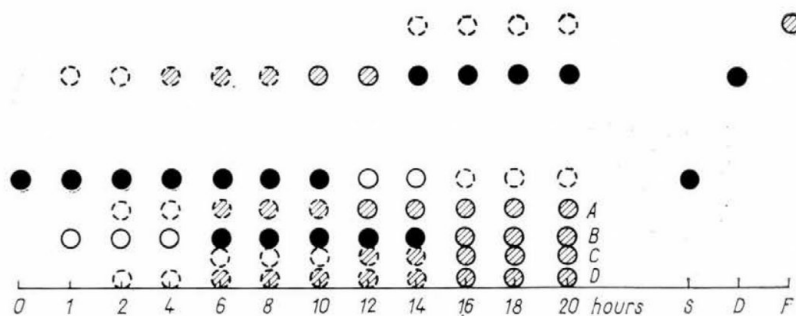


Fig. 1. Sucrose breakdown by 10 g acetonized mycelium of *Claviceps* sp. mutant Ix 1326; 17.1 g sucrose in 100 ml M/15, pH 5.5 phosphate buffer. Right hand side of chromatogram: sucrose glucose and fructose controls. Figures under the start line indicate the incubation period in hours

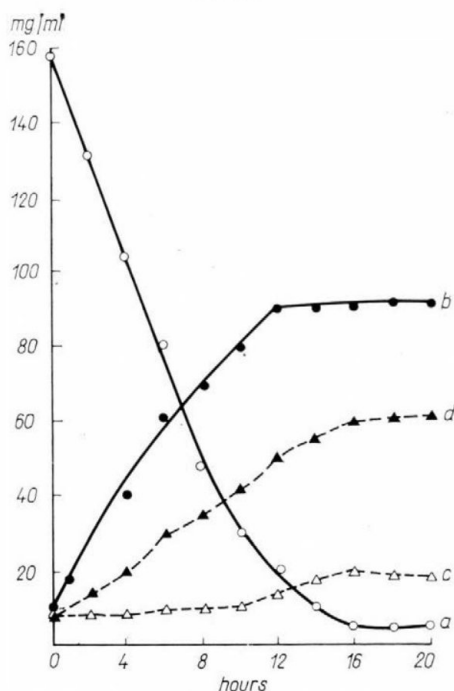


Fig. 2. Sucrose breakdown by 10g acetonized mycelium of *Claviceps* sp. mutant Ix 1326. Incubation system as indicated in Fig. 1. a) sucrose; b) reducing sugars; c) fructose; d) glucose

A, B, C and D, appeared on the chromatogram at different stages of incubation and were present at various concentrations (Fig. 1).

In case of strain T 20 only spot B was detected. Production of glucose and fructose was somewhat slower than that observed with strain Ix 1326.

Strain Se 1 produced carbohydrates A and B, but in considerably smaller amounts than did strain Ix 1326 and it produced glucose and fructose weakly,

similarly to strain T 20. Glucose appeared first and at higher concentrations than fructose.

In the above experiments the total reducing substance content of the system was examined parallel with chromatography. Strain Ix 1326, which displayed the strongest activity, was examined also for sucrose breakdown

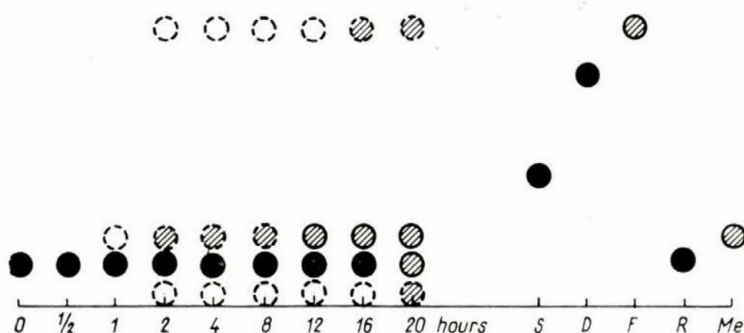


Fig. 3. Raffinose breakdown by 10 g acetonized mycelium of *Claviceps* sp. mutant Ix 1326; 10 g raffinose in 100 ml M/15, pH 5.5 phosphate buffer. Right hand side of chromatogram: sucrose, glucose, fructose, raffinose and melibiose controls. Figures under the start line indicate the incubation period in hours

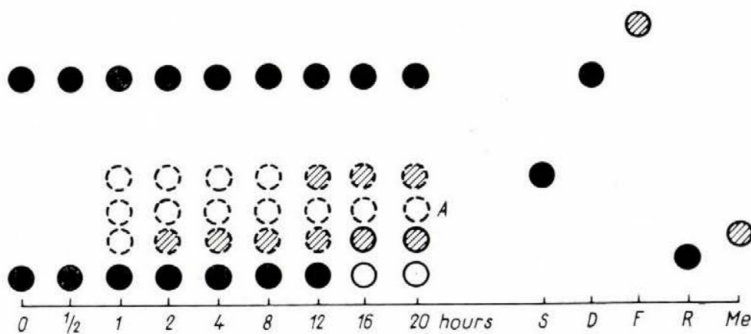


Fig. 4. Raffinose breakdown by 10 g acetonized mycelium of *Claviceps* sp. mutant Ix 1326 in the presence of glucose; 10 g raffinose and 10 g glucose in 100 ml M/15 pH 5.5 phosphate buffer. See Fig. 3 for further data

and glucose and fructose production (Fig. 2). These examinations could not be performed with the other two strains because their reducing substance production was less than 10 mg/ml in 20 hours. From Fig. 2 it is evident that with strain Ix 1326 there was a rapid increase in the total reducing substance content, approximately half of which was comprised by glucose. Fructose concentration increased slightly and only at the end of the incubation period.

In subsequent experiments we attempted to separate carbohydrates A, B, C and D detected on the chromatograms of strain Ix 1326. It was demonstrated that only carbohydrates A and C were able to reduce silver nitrate and TTC. Further experiments with parallel chromatograms showed that each of the four substances was detectable with anthrone.

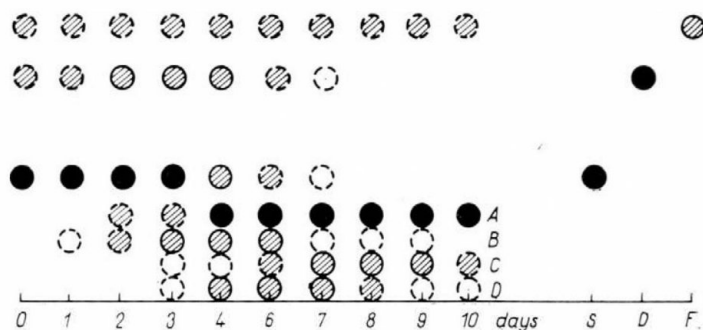


Fig. 5. Sucrose utilization by *Claviceps* sp. mutant Ix 1326 in a 10 litre glass fermentor. Six litres of Stoll medium supplemented with 17.1% sucrose and 10% inoculum; 0.5 litre air per litre medium per minute; stirring at 250 r.p.m. Right hand side of chromatogram: sucrose, glucose and fructose controls. Figures under the start line indicate the fermentation period in days

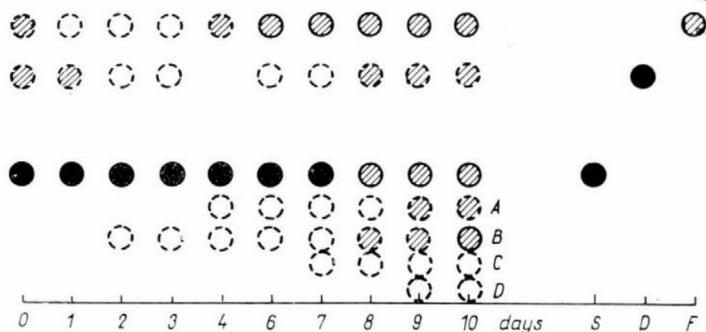


Fig. 6. Sucrose utilization by *Claviceps* sp. strain Se 1 in glass fermentor. For data, see Fig. 5

Carbohydrates A, B, C and D were separated from sucrose, fructose and glucose by column chromatography. The four substances were hydrolyzed with sulphuric acid then chromatographed. The examination showed the presence of glucose and fructose. The latter was shown in considerably higher amounts than the former. When the four substances were treated with BDH invertase, the same result was obtained as on acid hydrolysis.

Incubation of the four substances with acetone preparations of different invertase-producing yeast strains supplied the following results. *C. bever-*

wijkii caused the disappearance of C in 1 hour and of B in 4 hours; after 7 hours only glucose and fructose were detected. After 1 hour incubation with *C. pseudotropicalis*, carbohydrate B disappeared but the others remained well

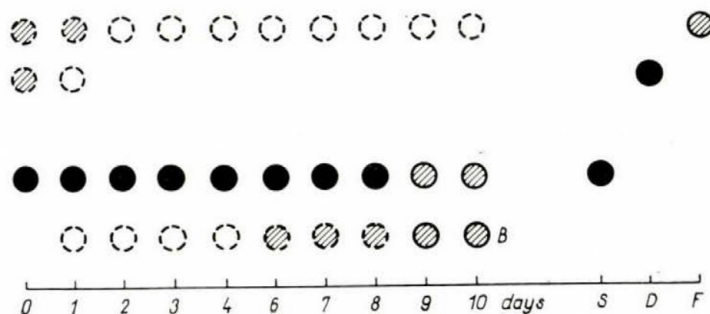


Fig. 7. Sucrose utilization by *Claviceps* sp. strain T 20 in glass fermentor. For data, see Fig. 5

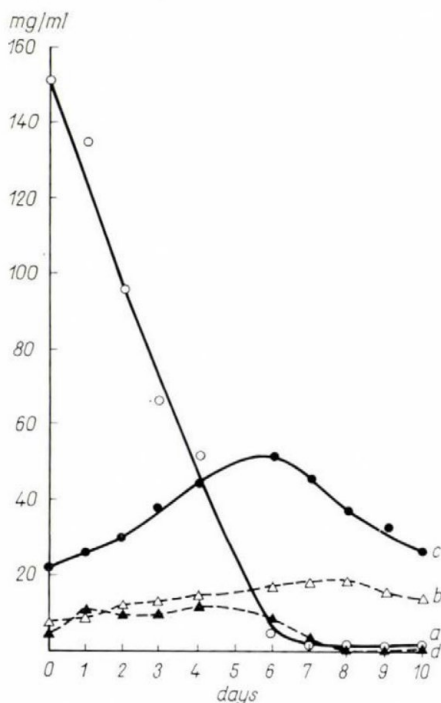


Fig. 8. Sucrose utilization by *Claviceps* sp. strain Ix 1326 in glass fermentor. For data, see Fig. 5. a) sucrose b) fructose; c) reducing sugars; d) glucose

detectable; after 2 hours only glucose and fructose were present in significant amounts. *S. cerevisiae* eliminated C within 1 hour, A and D within 2 hours and B within 4 hours. Incubation with the cell-free extract of endoglucosaccharase producing *C. reequinyii* produced no change in the chromatogram. The yeast strains liberated considerably larger amounts of fructose than of glucose.

When the acetonized mycelium of strain Ix 1326 was incubated in raffinose, in addition to melibiose and fructose, the gradual appearance of a spot lower in R_f value than raffinose was noted (Fig. 3). If the system was supplemented with 10 per cent glucose at the beginning of incubation, sucrose and carbohydrate A appeared instead of the mentioned spot (Fig. 4).

Glass fermentor experiments on sucrose hydrolysis in Stoll medium [13] by living mycelium supplied the following results.

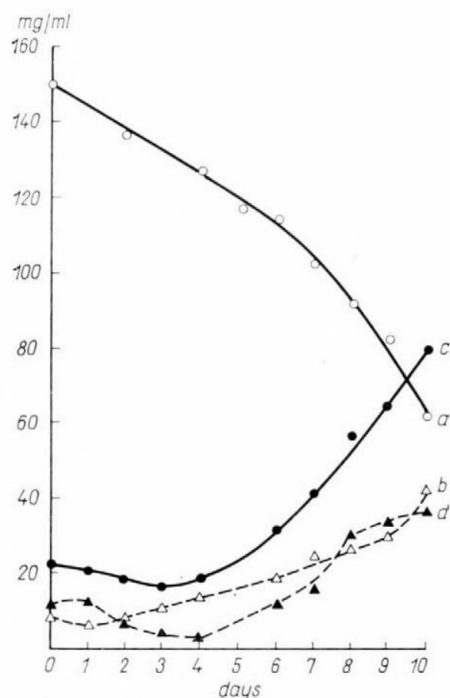


Fig. 9. Sucrose utilization by *Claviceps* sp. strain Se 1 in glass fermentor. a) sucrose; b) fructose; c) reducing sugars; d) glucose

Figs. 5—7 show the results obtained with the three different strains. Figs. 8—10 indicate the changes in sucrose, total reducing substances, glucose and fructose content in the case of the same strains. The presence of glucose and sucrose at the beginning of fermentation was a result of sucrose inversion during sterilization of the medium.

It is seen that there was a striking difference between the strains. With strain Ix 1326, the breakdown of sucrose and production of carbohydrates A, B, C and D took place very rapidly; finally, the latter products disappeared partly or totally. With strain Se 1 the same products appeared much slower, the sucrose content decreased but was not eliminated, and there was no final decrease in the concentration of the carbohydrates produced. Strain T 20

decomposed sucrose slowly, produced only substance B and was incapable of breaking down the latter. Quantitative conditions are clearly seen in the graphs.

Results obtained with the acetonized and living mycelium of strain Ix 1326 are compared in Fig. 11.

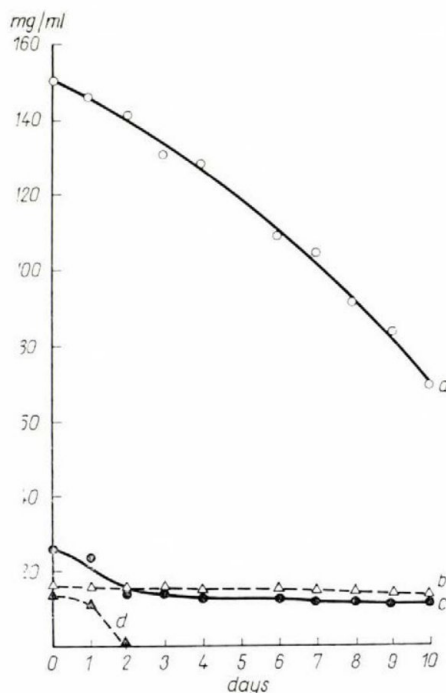


Fig. 10. Sucrose utilization by *Claviceps* sp. strain T 20 in glass fermentor. a) sucrose; b) fructose; c) reducing sugars; d) glucose

Discussion

Since the studies of BACON and EDELMAN [29] and BLANCHARD and ALBON [30] on the ability of yeast invertase to transfer fructosyl residue, several authors have examined various groups of yeast invertases in this respect. In filamentous fungi (*Aspergillus oryzae*), BEALING and BACON [31] were the first to demonstrate the fructosyl transferring capacity of invertase. Subsequent authors [32–37] showed and isolated various transfer products formed from sucrose by different fungi (*A. oryzae*, *A. niger*, *Penicillium spinulosum*, *P. chrysogenum*, etc.).

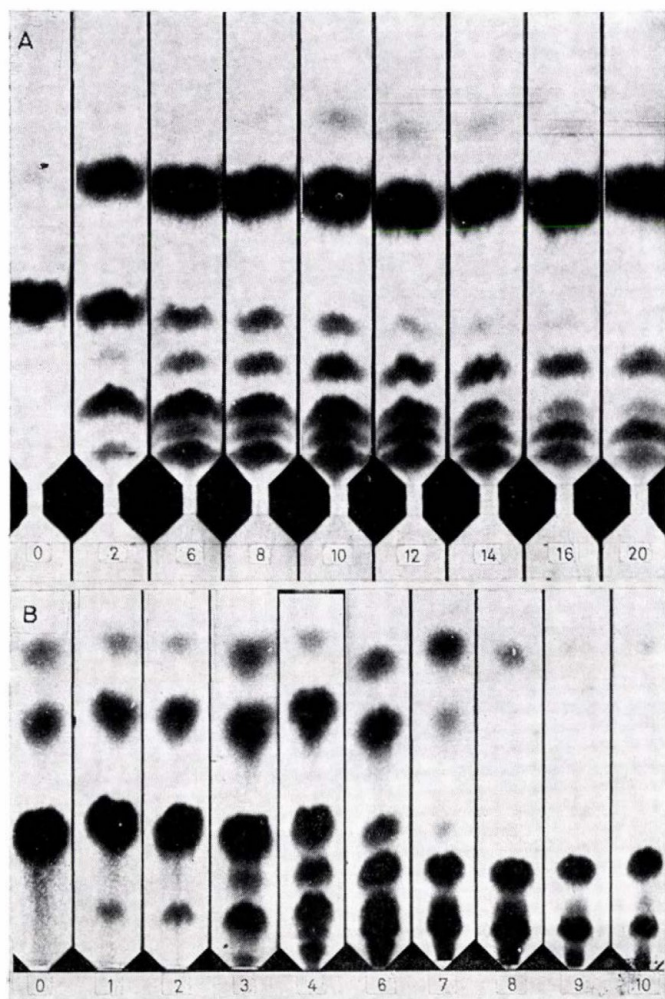


Fig. 11. Sucrose breakdown and utilization by *Claviceps* sp. mutant Ix 1326. A: sucrose breakdown by acetonized mycelium in shaken medium (see Fig. 1); B: sucrose utilization by living fermentor culture (see Fig. 5)

The following products have been identified: 1-inulobiosyl-glucose (beta-fructofuranosyl-2 — 1-beta-fructofuranosyl-2 — 1-alpha-glucopyranoside, kestose-1), 1-inulotriosyl-glucose (beta-fructofuranosyl-2 — 1-beta-fructofuranosyl-2 — 1-beta-fructofuranosyl-2 — 1-alpha-glucopyranoside). From raffinose the production of fructosyl raffinose and a non-identified pentasaccharide was observed [32]. It has been unanimously accepted that, with one exception, these substances differ from the products formed by yeast invertases. The latter have also been studied by several authors and, according to ALBON *et al.* [38] and GROSS *et al.* [39], correspond to the following compounds: kestose-1 (see above), kestose-6 (beta-fructofuranosyl-2 — 6-beta-

fructofuranosyl-2—1- α -glucopyranoside) and neokestose (beta-fructofuranosyl-2—6- α -glucopyranosyl-1—2-beta-fructofuranoside). There is another important difference between mold and yeast invertases: from sucrose, with the aid of a glucose acceptor, the former are able to resynthesize sucrose, while the latter form only 6-fructofuranosyl-glucose [40].

Sucrose decomposition by the *Claviceps* strains examined in this work can be summarized as follows.

(1) Each of the 3 strains produced invertase. This was confirmed by the fact that the strains caused extracellular sucrose and raffinose breakdown [13].

(2) As expected from data for filamentous fungi and on the basis of our previous studies [13], the examined strains produced transferring invertases.

(3) Number, amount and production order of the transfer oligosaccharides varied with each strain. Strain Ix 1326 produced large amounts of carbohydrates A, B, C and D. Strain Se 1 produced A and B in smaller amounts and C and D in traces. As compared to Ix 1326, with strain Se 1 the process was much slower and the carbohydrates appeared in different order. Strain T 20 produced substance B only.

(4) The properties of the transfer products were as follows.

(a) Two out of the four carbohydrates were reducing compounds (A and C).

(b) As shown with reagents selective for fructose-containing oligosaccharides, fructose was present in each of the four products.

(c) Each product contained linkages which were broken by yeast invertases (BDH invertase or acetonized preparations from different yeasts), but not by endoglucosaccharase (cell-free extract of *C. requinnyi* [26]). These results pointed to a beta-fructofuranosyl unit transfer during sucrose breakdown.

(5) It was remarkable that invertases in the three yeast strains decomposed the transfer products in the following order. *C. beijerinckii*: C, B; *C. pseudotropicalis*: B, C.; *S. cerevisiae*: C, A and D. These findings were indicative of small but detectable differences in the invertases of various yeasts.

(6) Invertase decomposition as well as acid hydrolysis have shown that the products contained more fructose than glucose. This finding indicated that glucose and sucrose, as well as their derivatives formed in primary transfer may act as acceptors in the transfer of fructosyl units.

The occurrence of fructosyl transfer was supported, in addition to observations of other authors, by our orientation experiments revealing that during sucrose breakdown methyl- and ethyl-betafructofuranoside are produced in media containing methanol and ethanol, respectively.

(7) The production in raffinose medium of a substance characterized by an R_f value lower than that of raffinose, was also indicative of the occurrence of fructosyl transfer. This was confirmed by the absence of a break in the

liberation of melibiose, and also by the observation that mold invertases are inducing transfructosidation. In the latter process another raffinose molecule is the acceptor and accordingly fructofuranosyl-raffinose is produced.

(8) It has been mentioned that glucose may act as an acceptor in sucrose breakdown. In such processes glucose is produced as a result of a low degree hydrolytic decomposition. This consideration has been proved on the one hand by the settling of an equilibrium in which the medium still contained sucrose and by a relative increase in sucrose concentration following the considerable initial decrease of this sugar. The assumption has been confirmed by the demonstration of sucrose production during raffinose breakdown in the presence of glucose. It should be emphasized that in this process the formation of the mentioned transfer product (probably fructosyl-raffinose) was prevented by applying the sugars in suitable concentrations (10% raffinose and 10% glucose).

(9) Observations on sucrose utilization by living mycelium and role of transfer products in sucrose metabolism are also worth mentioning. In this respect there were essential differences between the strains. In case of mutant Ix 1326 the production and subsequent disappearance of the transfer products occurred rapidly. With the other two strains the transfer products appeared slowly and their disappearance could not be demonstrated within the incubation period. Considering the results obtained with the acetone-organism it may be assumed that strain Ix 1326, in contrast to the others, contains large amounts of invertase, the majority of which acts outside the permeation barrier.

Accordingly, in view of the two possible pathways of sucrose utilization — the direct utilization of glucose liberated partly by hydrolysis and partly in transferring reactions, the indirect utilization of glucose incorporated passively into the transfer products, as well as the direct use of fructose liberated on hydrolysis — the capacity to and rate of free glucose and fructose assimilation are not indifferent. Assimilation of free sugars is the only way in which the sucrose-glucose-fructose-transfer product equilibrium developed by cleavages, transfers and resyntheses from the sucrose substrate can be broken.

Earlier findings indicating differences between the strains in this respect [13] were in agreement with the present results. Namely, strain Ix 1326, which had been found to assimilate glucose more effectively than the other cultures, was the only one capable of upsetting the equilibrium and assimilating by means of glucose utilization sucrose and its transfer products perfectly. In the two other strains the weak primary sucrose breakdown was associated with weak glucose assimilation. Thus not only the production of transfer oligosaccharides but also the upsetting of the slowly ensuing equilibrium was delayed. This condition, despite the satisfactory sucrose concentration, led finally to a physiological starvation. It should be noted that among our cultures only strain Ix 1326, which had been found satisfactory as regards primary

sucrose decomposition and upsetting of the equilibrium (glucose assimilation), was able to produce the alkaloid. It has been emphasized in the literature that alkaloid production is always associated with good sucrose assimilation. On the basis of the present findings this consideration can be supplemented by another observation, namely, that effective glucose assimilation is needed in addition to good invertase activity.

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PRODUCTION OF CLAVINE-ALKALOIDS BY CLAVICEPS FUSIFORMIS (LOVELESS) IN SUBMERGED CULTURE

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Summary. In previous work the relationship between the morphology of *Claviceps paspali* and *Claviceps purpurea* and the production of alkaloids in submerged culture was described. With these species alkaloids were produced only when the structure was sclerotial. With *Claviceps fusiformis* (Loveless) alkaloid production took place only with conidial strains. Natural sclerotia of *Claviceps fusiformis* showed in the sclerotium in contrary of other species of *Claviceps*, pear-shaped fruiting bodies containing large numbers of the two types of conidium. Systematic relationships of the different species of *Claviceps* and the preceding genus *Balansia* are discussed.

Several authors have described the production of clavine-alkaloids (agroclavine, elymoclavine, chanoclavine, etc.) in surface [1, 2, 3], and more recently also in submerged cultures [4]. Nevertheless the relations of morphology of mycelium developing on nutrient agar or in submerged culture to the production of alkaloids have not been examined.

Since such a relationship has been demonstrated for *Claviceps paspali* (Stev. et Hall) [5, 6] it seemed of interest to extend these studies to the strain *Claviceps fusiformis* (Loveless).

Materials and methods

Origin of the strain. A strain of *Claviceps* sp. No B. 35, kindly supplied by DR. J. RENZ, Sandoz Co., Basel, Switzerland, was used. It was isolated from sclerotia collected on *Pennisetum tiphoideum* Rich. The same strain in our collection has the identity number F-1307. This strain of *Claviceps* sp. belongs to *Claviceps fusiformis*, as has been stated in a recent paper by LOVELESS [7].

Culture media. (1) Agar medium. The culture was maintained on a potato-glucose medium [6]. Subisolations were made on plates in Petri dishes of 12 cm $\varnothing$ containing 15—20 ml of potato-glucose medium. (2) Fluid medium. Three different media were used for the submerged cultures. **Medium A:** Glucose potato broth. **Medium B:** Mannitol, 50 g; KH_2PO_4 , 1 g; MgSO_4 , 0.3 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.013 g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.004 g; succinic acid, 5.4 g; distilled water, 1000 ml; pH adjusted to 5.2 with NH_4OH [3]. **Medium C:** Sucrose, 100 g; peptone Difco, 20 g; distilled water, 1000 ml. The culture media were sterilized for 30 minutes at 105° C.

Fermentation methods. (1) Shake flasks. Cotton-wool plugged 500 ml Erlenmeyer flasks containing 100 ml culture medium were incubated at 27° C in a rotary shaker of 220 r.p.m. and an eccentricity of 10 cm.

(2) Inoculation. Shake flasks containing medium A were inoculated with vegetative mycelium grown on agar slant for 10 to 15 days. After 5—7 days the contents of one flask were

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homogenized in a Waring blender for 10 to 20 seconds and 10 ml of the suspension was used to inoculate shake flasks containing 100 ml medium A for the seed cultures.

The shake flasks containing fermentation medium B or C, were inoculated with 10 ml of the seed suspension grown for 24 or 48 hours.

Analytical methods. (1) Alkaloid. The clavine alkaloids were determined by VOIGT's colorimetric method, based on the formation of a blue colour with the EHRLICH—VAN URK reagent. A calibration curve was plotted using agroclavine as reference standard giving a linear plot in the range of 5 to 30 $\mu\text{g/ml}$. Results were expressed as $\mu\text{g/ml}$ of agroclavine.

(2) Dry weight. An aliquot of the mycelium suspension (usually 20 to 100 ml) was filtered through paper on a Buchner funnel, washed thoroughly with three volumes of water and dried at 85° C for 24 h.

(3) Microscopic examination. This was carried out on fresh mycelia, unstained or stained with cotton-blue in lactophenol. Lipids in the hyphae were demonstrated by staining with Sudan III in lactophenol. Nuclei were stained with the Giemsa method [5].

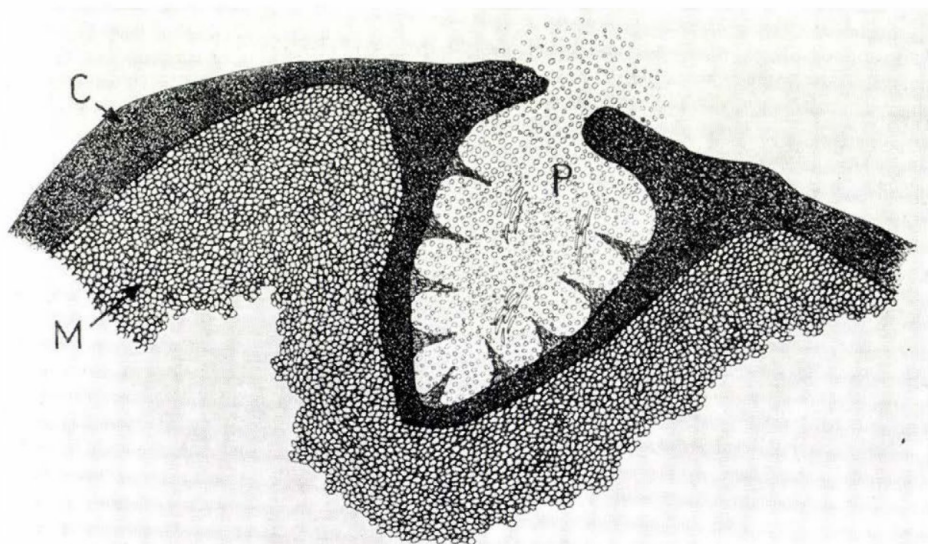


Fig. 1. Diagram of a cross section of *Claviceps fusiformis* sclerotium. C = cortex; M = medulla; P = fruiting body, conidia

Results

Morphological studies. (1) *Morphology of natural sclerotia.* Sclerotia obtained from the C. M. I. KEW, London collected on *Pennisetum tippoideum* Rich. (I. M. I. No 72492), and from DR. P. MANTLE (Department of Biochemistry, Imperial College, London) were available for morphological examination. They were oblong, oval, dark brown, with smooth surface, 3–4 × 1–1.5 mm in size. In thin sections they exhibited a dark-brown, 30–50 μ thick cortical part invaginating into the white-grey medullary part (Fig. 1). The plectenchymatic structure consisted of sclerotial cells, roundish, 6–16 μ in diameter, or rectangular and 15–20 × 4–8 μ in size containing fat droplets. Inside the sclerotium was seen the fruiting-body, similar in form and structure to picnidia, full of conidia (Fig. 1 and Fig. 2).

In thin sections the fruiting-body was pear shaped, $600-750 \times 300-480 \mu$ in dimension and was separated from the internal layer of the sclerotium by a $30-40 \mu$ thick dark-brown wall. The fruiting-body split outward with a cleft, enabling the conidia to escape (Fig. 1, Fig. 2).

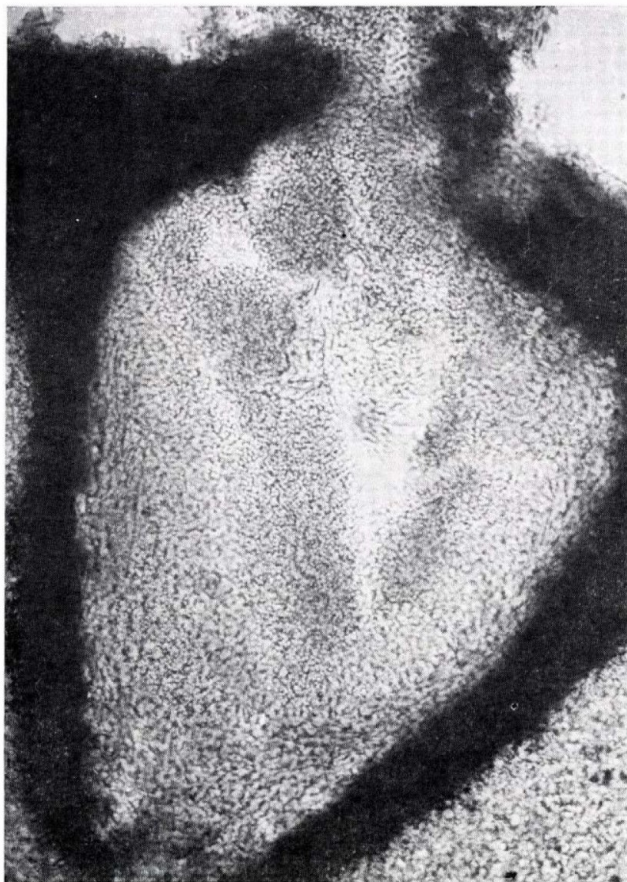


Fig. 2. Cross section of *Claviceps fusiformis* sclerotium (photograph)

In longitudinal sections the fruiting-body's shape was typically hysteroïd, indicating a relationship to the family *Discellaceae*.

The walls inside the fruiting-body showed, a conical apophysis measuring $120-150 \times 30-50 \mu$, penetrating into the central cavity. On the walls of this apophysis and on those of the fruiting-body there were conidiophores formed by a sterigma, $10-12 \times 3-4 \mu$, at whose apices the conidia were apparent (Fig. 3).

The conidia were of two types. Type A: roundish, sometimes oval conidia, $4-6 \times 3-4 \mu$, in size. Type: B long oval conidia measuring $20-30 \times 3-4 \mu$. The fruiting bodies showed either one or both types of conidia.

(2) *Morphology of sclerotia produced on embryo*. Embryos of caryopsis of wheat (var. Mentana) sterilized and grown on agar slants containing WHITE's nutrient medium, were infected before germination with conidia of the strain F-1307 of *Claviceps fusiformis* [8].

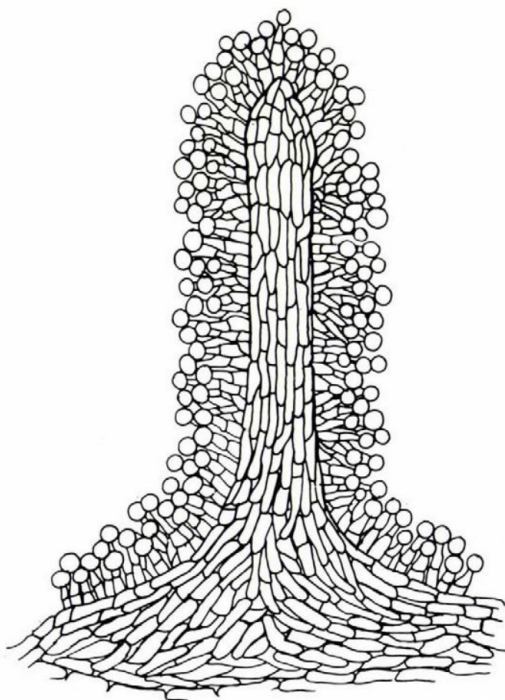


Fig. 3. Schematic presentation of an apophysis in the fruiting body

After twenty days, formation of sclerotia on the embryos took place in about 10%; the inoculated seeds showed the same structure as natural sclerotia (Fig. 4). Formation of fruiting body was observed on the surface and inside the sclerotium. In the latter case the fruiting-bodies resembled a roundish cavity, $200-300 \mu$ wide, full of conidia, separated from the sclerotium by a $30-40 \mu$ thick wall. In this case conidia Type A were present (Fig. 5).

(3) *Morphology of the original strain Claviceps fusiformis*. *Claviceps fusiformis* F-1307 did not appear homogeneous showing wide variability in both its colonies growing on nutrient agar and its alkaloid production in submerged culture.

After 12-15 days, the colonies attained a diameter of 1.5-2 cm with fluffy or wooly, whitish-grey aerial mycelium and with compact vegetative

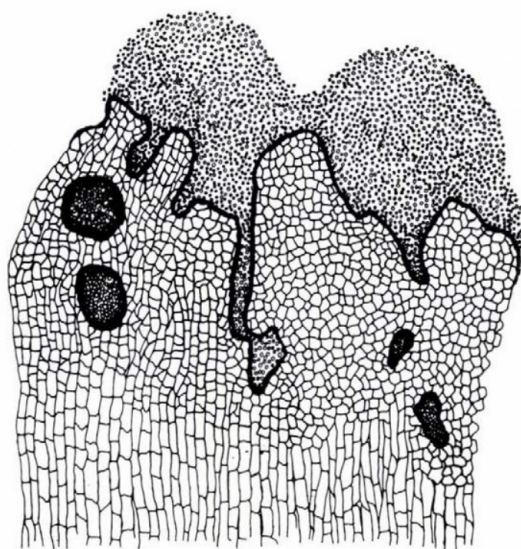


Fig. 4. Diagram of a cross section of artificial sclerotium of sterile germinated wheat

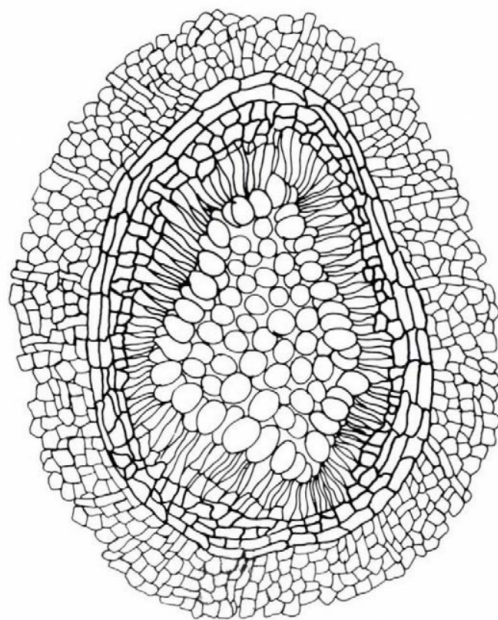


Fig. 5. Scheme of a round fruiting body from the sclerotium (detail of Fig. 4)

mycelium, sclerotial in structure and dark-brown or violet in colour. In general, this strain was very similar in morphology to that described later as colony type A.

(4) *Types of colony obtained by subisolation.* Extensive variability of the original strain necessitated subisolation from type A conidia. Colonies were distinguished as follows. *Colony type A.* These were roundish in shape, 1.5–2 cm in diameter on the 10th to 15th day, with continuous edge without or with very few fluffy white or greyish-white aerial mycelia. Vegetative mycelia were colourless. No conidia. In thin cross section the colonies showed inside, near the surface of the agar, a sclerotial prosenchymatic structure, 240–300 μ thick. The aerial mycelium presented a loose external structure showing hyphae 180–200 μ thick more or less bound together.

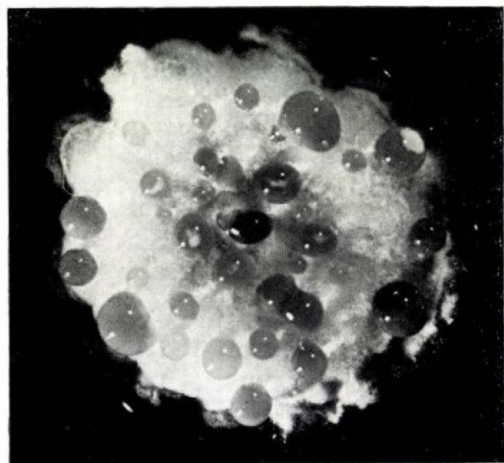


Fig. 6. B-type colony of *Claviceps fusiformis*

Colony type B. (Fig. 6) These were 1–1.5 cm large with whitish-grey aerial mycelia. The vegetative mycelium was dark-brown violet in colour. At the surface of young colonies many red-brown drops of honey-dew had formed which after 10–15 days were full of conidia. These drops were like those observed during the parasitic stage of the fungus under natural conditions (Sphaecelia stage). The structure of the colonies was sclerotial with plectenchima features. No loose layers were observed.

Both A and B types of conidium were present and they were formed in conidiophores contained by the fruiting-body. These fruiting-bodies may be outside the plectenchima and in this case they have a sinuous aspect valsoideous or dothideous; or are inside the plectenchima and then have a roundish form. These structures are very similar to those observed in natural sclerotia or in sclerotia obtained in embryos. The colonies produced a brown-violet pigment which diffused into the nutrient medium.

Colony type C. Colonies with continuous edges 1.8–2.5 cm in diameter, whitish-grey to violet aerial mycelium, and dark-brown violet vegetative

mycelium. Both an inner and an outer mycelial structure were present. Fruiting-bodies were found rarely and then just outside the roundish colony. No honey-dew drops were present. The main characteristic of these colonies was

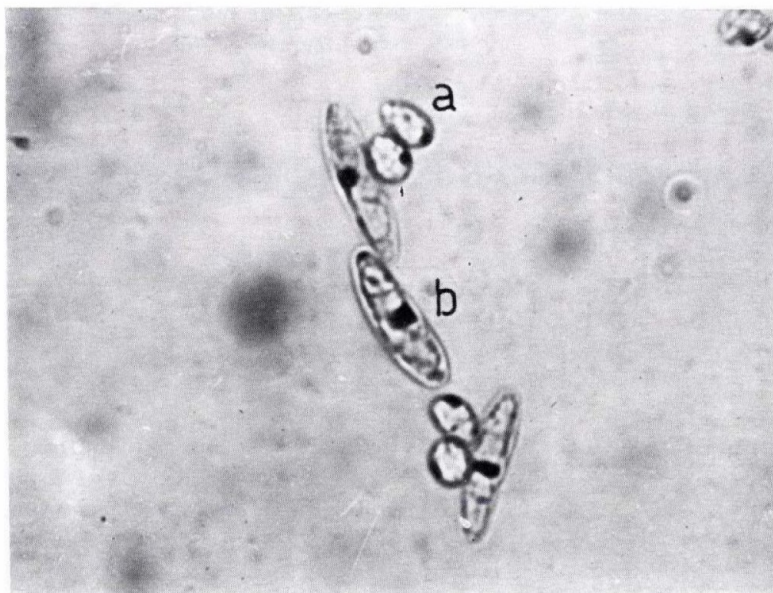


Fig. 7. Conidia of *Claviceps fusiformis*

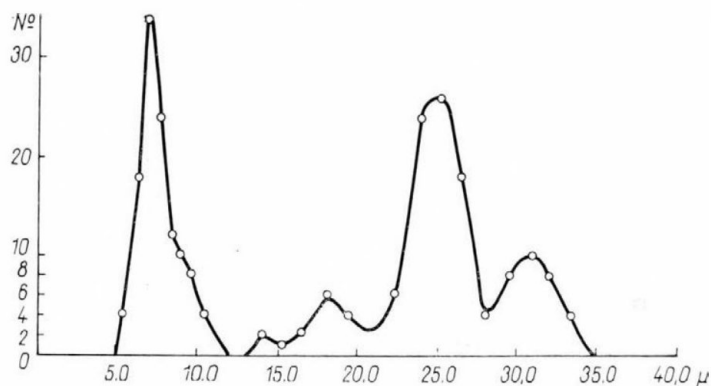


Fig. 8. Distribution of dimensions of conidia of *Claviceps fusiformis* grown on agar medium

the discontinuity in the hyphae which released their own cytoplasm. Pigment diffusing into the agar was also present.

(5) *Conidia of Claviceps fusiformis* F-1307 in saprophytic culture. Two types of conidium have been observed in cultures of *Claviceps fusiformis* F-1307, similarly to those occurring in natural sclerotia. Type A was represented

by roundish conidia $5-10\ \mu$ in diameter, with a single nucleus (Fig. 7/a). They originated from typical conidiophores formed by small sterigmata arisen directly from a vegetative hypha. Type B was characterized (Fig. 7/b) by large, long conidia $14-33 \times 4-6\ \mu$. Distribution of conidial length is shown in Fig. 8. It was almost impossible to isolate colonies containing a single type of conidium. The two types were always produced in mixed forms as occurs also in *Claviceps gigantea* [9]. Both types of conidium were formed also in submerged culture.

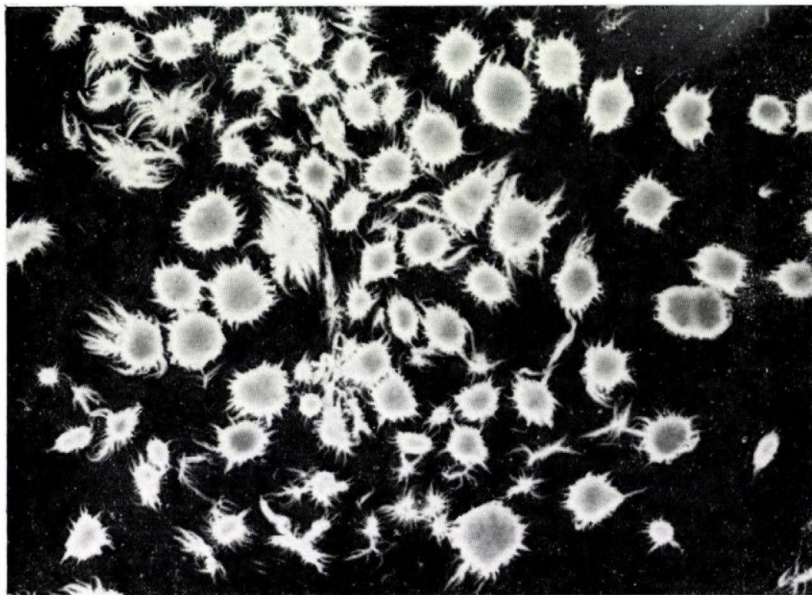


Fig. 9. Sinnematous pellet. Submerged culture of A-type colony

(6) *Morphology of the mycelium in submerged culture.* In submerged culture the mycelia showed different structures. (a) Synnematic pellets formed by a central pellet with the edge fringed by synnemata (Fig. 9). The mycelium was formed by straight hyphae without swelling cells and with a few fat droplets. The spheres reaching $4-5\ \text{mm}$ in diameter were white and no polysaccharide production took place during fermentation. This morphology appeared with type A colonies and no alkaloids were produced.

(b) Simple synnemata (Fig. 10) formed by interwoven hyphae measuring $3-4 \times 0.1-0.3\ \text{mm}$. The hyphae showed many swelling cells with clamidosporous structure. The mycelium was reddish brown, with many conidia. Pigment and polysaccharide were formed during fermentation. This morphology appeared with type B colonies and large amounts of alkaloid were produced.

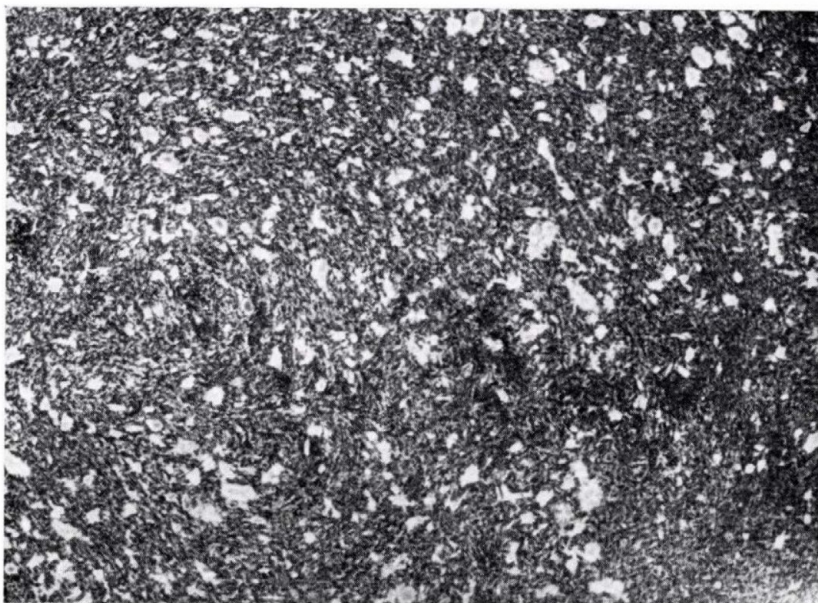


Fig. 10. Sinnema. Submerged culture of B-type colony

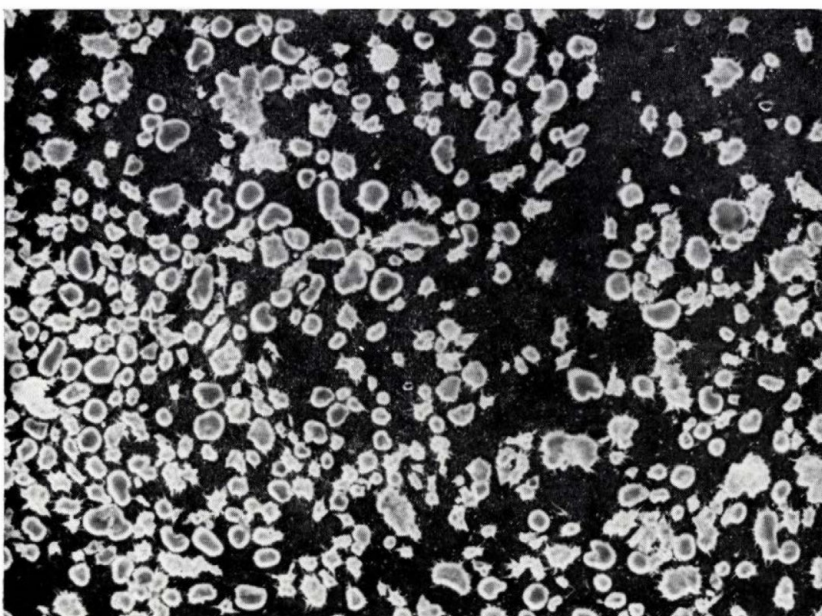


Fig. 11. Pellet. Submerged culture of C-type colony

(c) Pellets without synnemata (Fig. 11) dark-brown in colour not producing polysaccharides only some alkaloid during fermentation. No conidia were present. The hyphae exhibited swelling cells which disrupted and readily released their cytoplasm. This type appeared with type C colonies.

Fermentations. Optimal alkaloid production in submerged culture was obtained with colony B on nutrient medium C: glycerol 10%, peptone (Difco) 2%, in distilled water, pH 6.5–6.8 (Table I). On this nutrient medium alkaloid production reached about 1600 $\mu\text{g/ml}$ after 7 days, if as carbon source glycerol was present. With this substrate no polysaccharides were formed; therefore the aeration reached maximum efficiency (Table II).

Table I

Alkaloid production of different types of colony (on medium C after 7 days)

Types of colony	Alkaloid $\mu\text{g/ml}$
A	0
B	1280
C	800

Table II

Alkaloid production in medium B and C with various carbon sources

Nutrient medium	Concentration of carbon source, per cent	Carbon source				
		Sucrose	Glucose	Lactose	Mannitol	Glycerol
B	10	45	25	15	10	5
C	10	750	—	—	850	1600

Analysis after 7 days. Two experiments, each in triplicate, were performed.

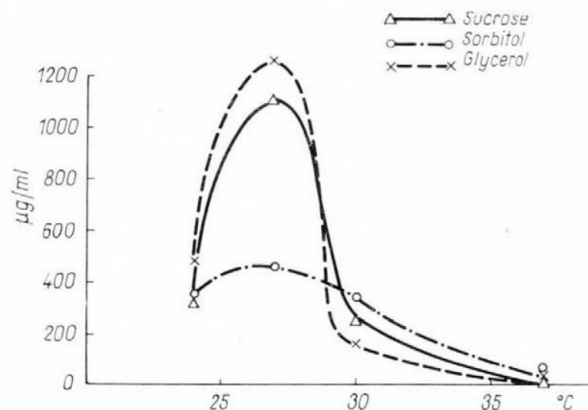


Fig. 12. Alkaloid production of *Claviceps fusiformis* grown at different temperatures and on different carbon sources

The optimal temperature was higher than the 27° C required by *Claviceps paspali*. The difference may have been due to the tropical or subtropical origin of *Claviceps fusiformis* (Fig. 12).

Discussion

Alkaloid production by *Claviceps paspali* in submerged culture occurs when the growing mycelium produces synnemata with a sclerotial structure like that of natural sclerotia. The same was found with *Claviceps purpurea* which produced peptide alkaloids in saprophytic culture only when the mycelium was of a sclerotial structure [10]. In fact, some strains of *Claviceps purpurea*, which produced conidia in agar slants did not produce alkaloids when grown in the same nutrient medium. The natural sclerotia of both *Claviceps* species showed a plectenchymatic structure and no conidia were present inside the sclerotium. With strain *Claviceps fusiformis*, alkaloid production reached the maximum only when the conidia were produced either on agar (colony type B) or in submerged culture. Only these colonies were similar to a true sclerotium; the natural sclerotia of this species contained fruiting-bodies which represented the Sphacelia stage of the species.

Claviceps fusiformis was clearly distinguishable by morphological features from *Claviceps purpurea* or *Claviceps paspali* but the relationship between alkaloid production and the morphological aspect of the natural sclerotium was still present.

The difference between strains *Claviceps purpurea* and *Claviceps fusiformis* was established also in saccharose cleavage, one of them containing beta-fructo-furanosidase, the other endosaccharase [11].

At present, it is impossible to explain the significance of the two types of conidium. In the genus *Balansia* which preceded the genus *Claviceps* in phylogenetic descent, the asexual stage was represented by picnidia related to the genus *Ephelis* (*Discellaceae*, *Scolecosporeae*) which were found in the same stroma containing perithecia [12]. Hence there is reason to assume that the fruiting-body inside the sclerotium of *Claviceps fusiformis* may be a subsequent stage of phylogenetic descent of this order. The long conidia resemble the *Scolecosporoides* conidia of the preceding genus.

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EFFECT OF CYTOSTATICS ON THE REPLICATION OF VACCINIA, PSEUDO-RABIES AND SINDBIS VIRUSES AND ON THE INTERFERON PRODUCTION BY TISSUE CULTURE CELLS

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Summary. The effect of several cytostatics has been examined on the replication of vaccinia, pseudo-rabies and Sindbis viruses and on the interferon production in chick embryo fibroblast (CEF) cell cultures. The replication of pseudo-rabies and vaccinia viruses was significantly inhibited by mannomustine, cyclophosphamide, mannityl dimensylate, 6-mercaptopurine, chlormethine N-oxide, cactinomycin, and pteropterin. Sindbis virus was inhibited only by mannomustine. The effect of these substances on interferon production was found to be a suitable method of assay of the in vitro toxicity of viral chemotherapeutics.

Replication of viruses in vitro is affected by several biologically active substances. Several metabolite analogons, antibiotics, alkylating agents and other substances have been shown to possess such an activity. Laboratory assays as well as field trials have, however, revealed that only some of these substances are sufficiently active to be considered candidates for viral chemotherapy. Nevertheless, examination of substances with sub-therapeutical activity seems to be useful, as it might give some clues for further research.

The present report deals with some cytostatics hitherto not examined for virostatic activity. Cactinomycin and 6-mercaptopurine were also included to examine the effectiveness in our system of these substances with proved antiviral effect.

An estimation of the action of the above compounds on interferon synthesis appeared to be of interest, as a possible inhibition of interferon production was expected to yield information concerning their interference with the aspecific protective reactions of the host cell to a viral infection. Interferon being a protein coded by cellular DNA [3], the inhibition of its production may point to a general impairment of protein synthesis.

A system consisting of vaccinia, pseudo-rabies or Sindbis virus and CEF cell cultures was used for studying the antiviral and interferon production inhibitory effects of a given cytostatic in one and the same system.

Materials and methods

Tissue culture. Cells were obtained by trypsinization of 11-day old chick embryos. The culture medium consisted of Gey solution containing 5% calf serum, 0.4% lactalbumin hydrolysate and 8% 0.1 M TRIS buffer pH 8.1. Tube cultures were seeded with 1 ml of a sus-

pension containing 1.5×10^6 cells/ml, Petri dishes (60 mm in diameter) with 5 ml of a suspension containing 5×10^6 cells/ml. Both types of cultures were incubated for 24 hours at 37°C before use.

Viruses. A strain of Sindbis virus, kindly supplied by Dr. I. GRESSER (Boston, Mass., USA), was maintained in this laboratory by serial passages in CEF cells. The pseudo-rabies virus was the strain maintained in CEF cells by Dr. I. BÉLÁDI in this laboratory. Vaccinia virus was obtained from the Phylaxia State Serum Institute (Budapest) and maintained by serial passages in HeLa cells.

Virus assay. All viruses used in the experiments were titrated as follows. Serial dilutions were prepared in PARKER's 199 medium (pH 7.4) and 0.5 ml volumes of the appropriate dilutions were allowed to adsorb to cell monolayers grown in Petri dishes for 1 hour at 37°C . This was followed by rinsing the cultures with 5 ml of the diluent medium. Cultures were then overlaid with a mixture of Gey solution, containing 0.25% lactalbumin hydrolysate, 5% bovine serum, 15% TRIS-HCl buffer (0.1 M, pH 8.1), 0.008% neutral red and 1.35% agar. The overlaid cultures were incubated at 37°C . Plaque counts for Sindbis virus, and for vaccinia and pseudo-rabies viruses were determined after 48 and 72 hours, respectively.

Cytostatics. Bayer E 39 [2,5-bis(ethyleneimino)-3,6-bispropoxy-1,4-benzoquinone; BAYER]; Degranol [mannomustine: 1,6-bis(β -chloroethylamino)-1,6-desoxy-D mannitol-2, HCl; CHINOIN]; Endoxan (cyclophosphamide: N,N-bis(β -chloroethyl)-N', O-propylenephosphoric acid ester diamide; BAYER); Mannitmyleran (mannityl dimesylate; 1,6-dimethylsulphonyl-D-mannitol; CHINOIN); 6-mercaptopurine (BAYER); Mitomen (chlormethine; N-oxide N-methyl 2,2'-dichlorodiethylamine N-oxide hydrochloride; BAYER); Myelobromol (1,6 dibromo-1,6-dideoxy 1,6-D-mannitol HCl; CHINOIN); Sanamycin (cactinomycin; actinomycin C₃; BAYER); Teropterin; (pteropterine; sodium pteroyl triglutamate; LEDERLE). From all compounds, stock solutions of 10 mg/ml concentration were prepared with distilled water. In case of need, they were adjusted to pH 7.4 either by 0.01 M NaOH or HCl. Further dilutions were prepared in nutrient medium.

Toxicity assay of cytostatics. Various concentrations of the individual cytostatics were added to CEF cell suspensions obtained by trypsinization. Tube cultures prepared from these suspensions and incubated for 4 days at 37°C were studied under the microscope for the degree of attachment to the glass, the extent of growth and the life span of the cells. Any difference in the above features observed between control and cytostatic-treated cultures was considered a toxic effect.

Assay of the virus inhibitory effect of cytostatics. Various dilutions of the individual cytostatics were added to tube cultures simultaneously with the viral inoculum (0.01 PFU per cell). Infected cultures were incubated at 37°C and the degree of virus reproduction was assessed by the plaque method [4] after 48 hours with Sindbis virus and after 72 hours with vaccinia and pseudo-rabies viruses.

Production and assay of interferon. Tube cultures of CEF cells were inoculated with doses of 0.1 PFU/cell of Sindbis virus and incubated for 48 hours at 37°C . The supernatants were then harvested and their virus contents inactivated by heat treatment (56°C , 60 min.). Twofold serial dilutions were prepared from the heat-treated material in PARKER's 199 medium (pH 7.4). One ml of each dilution was pipetted onto 24-hour CEF cell monolayers and incubated for 4 hours at 37°C . The cultures were then washed with 5 ml medium and infected with about 10^2 PFU Sindbis virus in 0.5 ml volume. Cultures were incubated for 2 hours at 37°C . After removal of the fluid, the monolayers were overlaid with agar medium. The titre of interferon was assessed by plaque counting after 48-hour incubation at 37°C . The interferon dilution reducing the plaque count by 50% was considered one unit.

Assay of the effect on interferon synthesis. Various dilutions of the test substances were added to CEF cell tube cultures simultaneously with inoculation of the virus, and the produced amount of interferon was estimated by the method described above. In these studies the Sindbis virus was used throughout.

Results

Toxicity of cytostatics. Results are presented in Table I.

Table I

Toxic concentrations of cytostatics

Substance	$\mu\text{g/ml}$
Bayer E 39	10
Mannomustine	15
Cyclophosphamide	400
Mannityl dimesylate	200
6-mercaptopurine	200
Chlormethine N-oxide	20
Myelobromol	200
Caactinomycin	0.1
Pteropterin.....	800

Inhibition of interferon production. To examine the inhibitory effect of the substances on interferon production 50% of the cytologically toxic concentration was applied as maximum test concentration. As shown in Table II, lower concentrations also suppressed interferon production. This observation suggested that the examined substances had impaired the metabolism of tissue culture cells at concentrations considerably lower than the microscopically detectable toxic level.

Effect on the replication of vaccinia, pseudo-rabies and Sindbis viruses. As shown by the experiments on the suppression of interferon production, the effective toxicity of the cytostatics was much lower than that detectable by cytological methods. Therefore, in our studies on antiviral activity the cytostatic agents were applied at concentrations lower than the smallest dose inhibiting interferon synthesis. The degree of virus inhibition was expressed in terms of the difference between log PFU of the control ($\log N^0$) and that in the presence of the inhibitor ($\log N$). Deviations of 0.1–0.3 exponents having been found in parallel experiments, values lower than 0.5 were not considered. The dilution series for each substance was extended to the level producing inhibition less than 0.5 exponent for all 3 viruses tested. The degree of inhibition of vaccinia, pseudo-rabies and Sindbis virus replication by the various cytostatics is presented in Table III.

With vaccinia and pseudo-rabies viruses, measurable inhibition was produced by all the tested cytostatics except BAYER E 39 and Myelobromol. The replication of Sindbis virus was inhibited exclusively by mannomustine. Effectiveness and activity range of mannomustine, cyclophosphamide, mannityl

Table II

*Effect of cytostatics on interferon synthesis
in Sindbis-virus-infected chick embryo fibroblast cells*

Preparation	$\mu\text{g/ml}$	Interferon titre
Control	—	64
Bayer E 39	0.62	64
	1.22	34
	2.5	8
	5.0	0
Mannomustine	2.5	64
	5.0	64
	7.5	16
Cyclophosphamide	10	64
	100	64
	200	16
Mannityl dimesylate	10	64
	50	64
	100	16
6-mercaptopurine	10	64
	50	16
	100	4
Chlormethine N-oxide	2.5	64
	5.0	64
	10.0	8
Myelobromol	25	64
	50	32
	100	4
Cactinomycin	0.012	64
	0.025	64
	0.05	32
Pteropterin	50	64
	100	64
	200	16
	400	0

Table III

*Effect of cytostatics on replication of vaccinia, pseudo-rabies
and Sindbis viruses*

Preparation	$\mu\text{g/ml}^*$	Logarithm of inhibition ($\log N^0 - \log N$)		
		Vaccinia	Pseudo-rabies	Sindbis
Bayer E 39	0.62	0.1	0.3	0.1
Mannomustine	0.62	0.5	0.9	0.1
	1.25	1.5	2.1	0.2
	2.5	1.8	2.6	0.2
	5.0	2.0	3.1	0.9
	10	2.0	3.1	0.9
Cyclophosphamide	5	0.3	0.4	0.1
	10	1.1	0.8	0.1
	100	1.4	1.9	0.2
Mannityl dimesylate	2.5	0.2	0.4	0.1
	5	1.2	1.4	0.1
	10	1.5	1.7	0.2
	50	1.8	2.0	0.2
6-mercaptopurine	2.5	0.2	0.3	0
	5	0.9	1.5	0.1
	10	2.1	2.0	0.1
Chlormethine N-oxide	2.5	0.2	0.4	0.1
	5.0	1.9	2.0	-0.1
Myelobromol	25	0.2	0.5	0.2
Cactinomycin	0.012	0.2	0.3	0.1
	0.025	1.2	1.7	0.1
Pteropterin	50	0.5	0.3	-0.1
	100	2.2	1.7	-0.2

* The cytostatic concentrations shown here had no influence on interferon production

dimesylate, chlormethine N-oxide and pteropterin were very similar to those of 6-mercaptopurine and cactinomycin.

Discussion

The cytostatics examined in the present study have been known to impair the normal metabolism of cells at relatively low concentrations. Their inhibitory effect on interferon production clearly supports the above observation as in the system used it could definitely be excluded that the inhibition of interferon production was the result of a suppression of the replication of the inducer virus (Sindbis virus). In Table IV are presented the effects of the test substances on the replication of Sindbis virus at concentrations shown to be

Table IV
Effect of cytostatics on replication of Sindbis virus

Preparation	$\mu\text{g/ml}^*$	Logarithm of inhibition ($\log N^0 - \log N$)
Bayer E 39	1.22	0.2
	2.5	0.2
	5.0	0.3
Mannomustine	7.5	1.1
Cyclophosphamide	200.0	0.3
Mannityl dimesylate	100.0	0.2
6-mercaptopurine	50.0	0.2
	100.0	0.2
Chlormethine N-oxide	10.0	-0.2
Myclobromol	50.0	0.3
	100.0	0.5
Cactinomycin	0.05	0.1
Pteropterin	200.0	-0.2
	400.0	-0.2

* The cytostatic concentrations shown here had decreased the production of interferon.

subtoxic by microscopic examination, but inhibitory to interferon production. At that concentration none of the cytostatics tested but mannomustine had any measurable effect on the replication of Sindbis virus. A comparison of the data in Tables I and II reveals that the lowest concentrations suppressing interferon production were 2–4fold lower than the cytologically toxic concentration. Consequently, the examination of the suppression of interferon production appeared to be a more sensitive method for toxicity assay than the microscopic examination of the cell morphology.

Replication of vaccinia and pseudo-rabies virus was inhibited not only by 6-mercaptopurine and cactinomycin, but also by mannomustine, cyclophosphamide, mannityl dimesylate, chlormethine N-oxide and pteropterin. Considering the ratio of inhibitory and toxic concentrations, none of these compounds appeared to be possible candidates as viral chemotherapeutics. It seemed to be of some interest, however, that only the two DNA viruses were inhibited considerably, while the RNA containing Sindbis virus was suppressed slightly or not at all. From these studies it cannot be judged how far this statement might be generalized for DNA and RNA viruses. The more so, as FREEMAN *et al.* [1] have shown that some alkylating agents were suppressing replication of RNA viruses within the test set vaccinia, polyoma, Rous sarcoma and EMC.

The point of attack of the alkylating agents mannomustine, cyclophosphamide, mannityl dimesylate and chlormethine N-oxide in the replication process of vaccinia and pseudo-rabies viruses has not been studied. Pteropterin, 6-mercaptopurine and cactinomycin have, however, been known to inhibit directly or indirectly the DNA-dependent RNA synthesis in various systems [3, 5, 6, 7, 8, 9]. According to REICH *et al.* [2], this results in a definite inhibition of the DNA virus vaccinia, but fails to affect the replication of the RNA viruses poliomyelitis and Mengo. The alkylating agents, though having been known to have numerous intracellular sites of attack [8, 10, 11, 12], displayed a greater affinity to DNA than to RNA and proteins [10, 13, 14, 15, 16, 17]. GRUNICKE *et al.* [18] suggested that the cytostatic action of the alkylating agent triaziquone results mainly from a suppression of DNA-dependent RNA synthesis. In our experiments, 6-mercaptopurine, pteropterin and cactinomycin inhibited the same viruses as did mannomustine, cyclophosphamide, mannityl dimesylate and chlormethine N-oxide, thus on the basis of literary data we have supposed that the inhibitory effect on vaccinia and pseudo-rabies viruses of the alkylating cytostatics tested was either the result of a suppression of the DNA-dependent RNA synthesis or that of a direct inhibition of viral DNA replication.

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SIGNIFICANCE OF STAPHYLOCOCCAL FATTY ACIDS IN THE PATHOGENESIS OF INFLAMMATION

By

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Summary. The lipid content of the mouse kidney and lung has been examined at various intervals after infection with *Staph. aureus*.

(i) Accumulation of free fatty acids in the kidney was proportional to the severity of inflammation. The increase in fatty acid content was three to sixfold as compared to the normal value.

(ii) The main component of free fatty acids was oleic acid (cis-vaccenic acid). The compact variant of *Staph. aureus* strain Smith gave rise to a two-fold increase in the amount of this substance: the increase was sixfold after a severer infection caused by the diffuse variant of the same organism.

(iii) Free fatty acids showed threefold increase in the lung of intrapleurally infected mice.

(iv) The importance in the pathogenesis of inflammation of fatty acid accumulation has been discussed.

The lipid constitution of bacteria depends considerably on the environment. When grown *in vivo*, the unsaturated fatty acid content of the organism increases. The present work has been undertaken to examine the importance of fatty acid synthesis in the pathogenesis of staphylococcal infections. The kidneys of mice developing nephritis after infection with the diffuse and compact variants of *Staphylococcus aureus* strain Smith were examined for lipid content with special regard to hexane-extractable lipids and free fatty acids. Changes in the lipid composition of the lung after intrapleural infection were also examined.

Materials and methods

Organisms. The diffuse and compact variants of *Staph. aureus* strain Smith were used. The bacteria were cultured on synthetic Difco casitone medium for 18 hours.

Mice. White mice (Humán, Budapest) weighing 18 to 20 g were infected intravenously with 0.1 ml bacterial culture suspended in saline. The suspension contained 6×10^6 cells per ml for the diffuse variant and 6×10^8 cells per ml for the compact variant. Three days after infection one drop of blood was taken from the caudal vein and cultured. On the 5th day urine samples were cultured in order to show renal infection. When neither the blood nor the urine samples gave positive cultures, the animals were reinfected.

The mice were divided into 3 groups with 15 animals each. On the 7th, 14th and 21st day after infection all animals in the corresponding group were killed with lighting gas. The kidneys were removed and stored at -20°C . Each experiment was carried out with the homogenized organs of one group of mice (15 animals).

Extraction of lipids. The organs were homogenized with pH 6 phosphate buffer in a Potter homogenizer. The homogenate was shaken out 3 times with 40 ml n-hexane, then the fractions were united and the water was removed. The solvent was then evaporated in nitrogen atmosphere under reduced pressure. The extracted lipids were dried over P_2O_5 and weighed.

Lipid analysis was carried out with thin layer and gas chromatography [7, 8].

Intrapleural infection of mice. White mice weighing 18 to 20 g were divided into groups containing 20 animals each and infected intrapleurally with 0.1 ml aliquots of the bacterial suspensions mentioned above. The animals were sacrificed 48 hours after infection, the lungs were frozen in liquid air then homogenized in the Potter apparatus. The homogenates, each containing the lungs of 20 animals, were subjected to lipid extraction with hexane as described above.

Results

At the end of the first week autopsy revealed hyperaemia but no other change in the majority of the organs. Two weeks after infection the kidneys of mice infected with the diffuse variant showed the presence of multiple abscesses varying in size. Liver abscesses were revealed in one animal. In 3 mice pulmonary congestion pointed to the infection. The spleen was generally soft and slightly enlarged. After 3 weeks renal changes became definite. Abscesses were formed in the kidneys of mice infected with both staphylococcal variants.

As compared to animals infected with the compact variant, gross changes were more definite in the mice inoculated with the diffuse variant. Similar morphological findings have been described in cases infected with the same bacteria [3-5].

The extractable lipid content in the kidneys of mice sacrificed 3 weeks after infection was considerably less than in the control group (Table I).

Table I

Lipid content of mouse kidney tissue after infection with the compact and diffuse variants of Staph. aureus strain Smith

	Dry weight percentage of lipids extracted with n-hexane		
	Compact variant	Diffuse variant	Control
Mice sacrificed 3 weeks after infection	7.1	8.4	16.0

The extractable lipids were subjected to thin layer chromatography in a petroleum ether: diethylether: acetic acid (82 : 18 : 1) solvent. The spots were detected in iodine vapour. In each experiment a 500 µg amount of the extract was chromatographed.

Fig. 1 demonstrates the thin layer chromatogram of mouse kidney tissue lipids extracted with n-hexane.

The main component was represented by triglycerides, which comprised about 80% of all extracted lipids. In addition, mono and diglycerides, phospholipids and free fatty acids occurred in well-detectable amounts. The chromatograms for infected and control mice differed considerably in the size of spots corresponding to free fatty acids. These substances were, therefore, determined quantitatively (Table II).

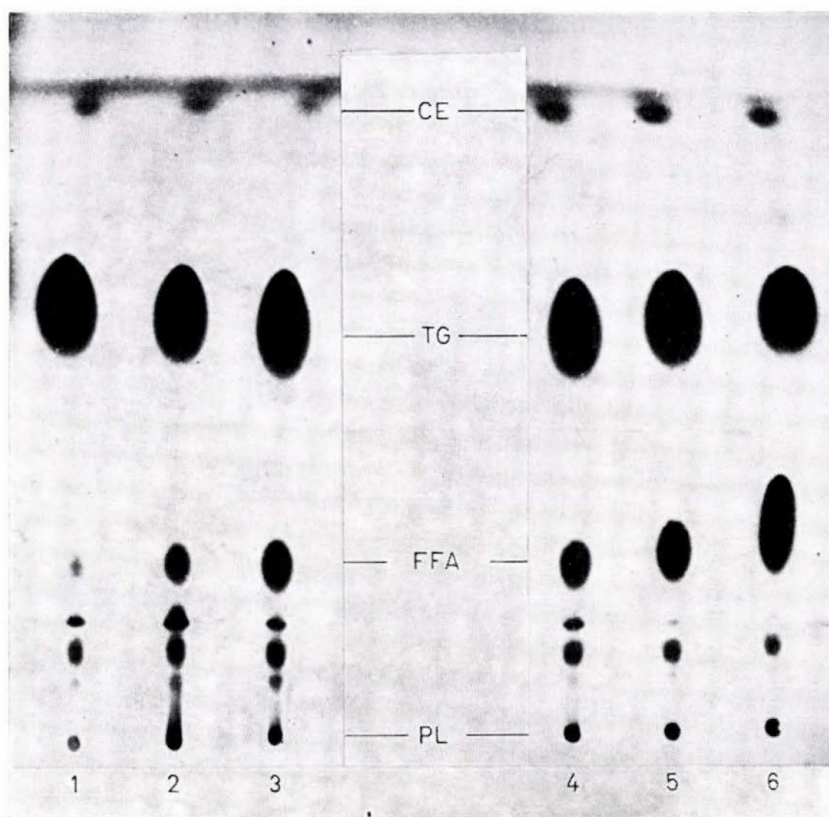


Fig. 1. Thin layer chromatogram of lipids extracted with n-hexane from mouse kidney tissue. Extracts: 1 = normal mice, 2 = mice infected with the compact variant and sacrificed after one week; 3 = mice infected with the diffuse variant and sacrificed after one week; 4 = mice infected with the compact variant and sacrificed after 3 weeks; 5 = mice infected with the diffuse variant and sacrificed after 3 weeks; 6 = extract 5 + oleic acid. Abbreviations: CE = cholesterol esters; TG = triglycerides; FFA = free fatty acids; PL = phospholipids. Thin layer: Silicagel G, 1000 μ . Detection of spots: iodine vapour

Table II

Free fatty acid content of mouse kidney tissue after infection with the compact and diffuse variants of Staph. aureus strain Smith

	Free fatty acid content in percentage of n-hexane- extractable lipid fraction		
	Compact variant	Diffuse variant	Control
Mice sacrificed 1 week after infection	3.8	5.7	1.3
Mice sacrificed 3 weeks after infection	3.6	8.0	

From Table II it is evident that the kidneys of mice infected with the staphylococcal strains contained free fatty acids in significantly higher amounts than the kidneys of the control animals. As compared to the control, the free fatty acid content was 3 times higher in the slight inflammation developing after infection with the compact variant. In the severe inflammation caused by the diffuse variant, the rise in free fatty acid content was 4.5-fold on the first week and 6-fold on the third week.

Components of the free fatty acid fraction were estimated by gas chromatography (Table III).

Table III
*Percentage distribution of free fatty acids
in mouse kidney tissue after infection with the compact
and diffuse variants of Staph. aureus strain Smith*

Fatty acids	Compact variant		Diffuse variant		Control
	Mice sacrificed after		Mice sacrificed after		
	1 week	3 weeks	1 week	3 weeks	
C ₁₂	10	9	9	9	10
C ₁₄	4	4	2	2	8
C _{16:1}	9	11	11	8	10
C ₁₆	14	18	14	13	19
C _{18:2+3}	4	4	5	5	8
C _{18:1}	37	41	45	49	29
C ₁₈	6	3	4	4	6
Others	16	10	10	10	10

It is seen that the main component was C_{18:1} (oleic acid or cis-vaccenic acid). The substance was present in larger amounts in severe diffuse variant infections than in mild changes caused by the compact variant. Thus, accumulation of oleic acid went parallel with the severity of inflammation. The amount of C_{18:1} fatty acid calculated for 100 g dry weight of kidney tissue of infected and control mice is presented in Table IV.

Table IV
*Free C_{18:1} fatty acid content in mg per 100 g dry mouse kidney
tissue after infection with the compact and diffuse variants
of Staph. aureus strain Smith*

	C _{18:1} fatty acid content	
	1 week after infection	3 weeks after infection
Compact variant	131	105
Diffuse variant	475	329
Control	63	

It is evident that, as compared to the control, both staphylococcal variants caused infections resulting in a considerable rise in $C_{18:1}$ fatty acid content.

The rise was about twofold after infection with the compact and about eightfold after infection with the diffuse variant. At the end of the third week the values decreased by 20 to 30%.

Virulent microorganisms are rapidly multiplying in the kidney and give rise to abscesses especially in the cortex. The animals die of the extensive destruction of renal tissue. Abscesses caused by less virulent strains gradually become sterile and then disappear.

Table V

Free fatty acid content of mouse lung tissue after infection with Staph. aureus

	Free fatty acid content in percentage of total lipid fraction	Saturated fatty acids per cent	Unsaturated fatty acids per cent
Infected mice	3.9	45	55
Control mice	2.1	61	39

Table V shows the saturated and unsaturated fatty acid content of the fatty acid fraction extracted from the lungs of mice infected intrapleurally with *Staph. aureus*.

The 2.1% free fatty acid content in the control animals' lung consisted of unsaturated fatty acids to 39 per cent. In contrast, the almost double amount (3.9 per cent) of free fatty acid content in infected mice yielded 45% saturated and 55% unsaturated fatty acids. The results also indicate that the unsaturated fatty acid content of the infected lung tissue was about threefold of the value obtained in normal lungs.

The change in the saturated:unsaturated fatty acid ratio in favour of unsaturated fatty acids indicated that the latter are responsible for the increase of fatty acid content.

Discussion

Biochemical changes occurring in the host after infection have not yet been elucidated. The present experiments indicate that fatty acids accumulate in tissues affected with inflammation.

As to the origin of the accumulated fatty acids there are only hypotheses. These substances may derive partly from bacteria partly from tissue lipids decomposed by lipolytic bacterial enzymes.

Among bacterial esterases, as described by ALFORD, PIERCE and SUGGS [1], staphylococcal enzymes are especially active on beta fatty acids of phospholipids. As these substances are mostly unsaturated compounds, unsaturated

fatty acids accumulating in inflammatory changes may be of tissue origin. It cannot, however, be excluded that unsaturated fatty acids produced in increased amounts by bacteria *in vivo* appear in the tissues [6].

Fatty acids may play a multiple role in the pathogenesis of inflammation. They may increase permeability by causing a change in pH and giving rise to an endothelial lesion; on the other hand they may promote the action on the tissues of toxic bacterial metabolites or may exert a direct injurious effect on the host cell.

There are few data for the role in biological functions of *Staph. aureus* lipases and the strain's pathogenicity. SHAH and WILSON [2] showed that the so-called "egg yolk factor" of *Staph. aureus* was a lipase-type enzyme. It hydrolyses the lipoproteins of intracellular structures and is, therefore, a significant virulence factor. The results of our experiments have supported this view.

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VIROLOGICAL EXAMINATION OF CHILDREN WITH ACUTE EXANTHEMATOUS DISEASE

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Summary. Two hundred and thirty-six children admitted with acute exanthems to isolating departments in the period from October, 1965, to November, 1966, were examined for bacteriological and virological infections. Virus isolation was attempted from the throat secretion and from the faeces in primary monkey kidney cell cultures, in the monkey kidney cell line III/1, in primary human embryonic kidney cell cultures and in cultures of the RK13 and HeLa cell lines. Paired sera were available from 159 cases. In the serum samples, depending on their available volume, the following antibodies were titrated: antistreptolysin, measles anti-haemagglutinin, complement-fixing antibodies to the group-specific adenovirus antigen and anti-rubella neutralizing antibodies. In addition, if virus had been isolated, the neutralizing antibody titre to the isolated virus was determined.

Based on the clinical picture, the 236 cases were divided into the following four groups: scarlet fever: 88 cases (37%), measles: 63 cases (27%), varicella: 46 cases (19%) and atypical exanthems: 39 cases (17%).

In the scarlet fever group *Streptococcus haemolyticus* infection was verified in 24%. Six cases proved to be measles, another six rubella. In further 7, 10 and 1 cases adenovirus, enterovirus and type 3 parainfluenza virus infection, respectively, was demonstrated.

In the measles group the diagnosis was verified in 30%. In 45% of the cases measles was excluded by a negative haemagglutination-inhibition test in the convalescent-phase sera. In 2, 5 and 14 of these seronegative cases rubella, adenovirus infection and enterovirus infection, respectively, was demonstrated.

In the varicella group *Herpesvirus varicellae* was isolated in the monkey kidney cell line III/1 from 12 cases, in RK13 cell cultures from 10 cases. The diagnosis was verified in 46% by virus isolation and/or neutralizing antibody response.

Out of the cases with atypical exanthems 3 proved to be rubella, 1 measles. In further 4, 3, 2 and 1 cases coxsackievirus, adenovirus, herpes simplex virus and RS virus infection, respectively, was demonstrated. In 3 cases *Str. haemolyticus* infection was present.

As a control group 222 non-exanthematous patients of similar age distribution were examined with the same tests.

Based on the enterovirus excretion associated with homologous seroconversion, an aetiological relationship is suggested between these viruses and the corresponding illnesses. The question whether the adenovirus infections were primary or secondary in character has remained unanswered.

In the last few years an increasing number of reports has discussed the aetiology of acute exanthematous diseases of children. Some authors analysed epidemics, others summarized their continuous studies carried out during a given period. The greatest interest has been attracted by the so-called atypical exanthems which are difficult to classify clinically. These studies have confirmed the earlier assumption that infection with certain enterovirus types [1—18], occasionally respiratory viruses [19—27], may be accompanied by cutaneous manifestations especially in children. The exanthem varies in character suggesting that, by a still unknown mechanism, the same type of virus may cause

rubelliform, morbilliform, or even vesicular, or urticarious exanthems. On the other hand, the same type of rash may be due to different viruses. Consequently, the aetiological factor often cannot be ascertained without virological examination.

The changes in the classical clinical picture of scarlet fever and measles, due to penicillin therapy and gamma globulin prophylaxis, respectively, resulted in further difficulties in differential diagnosis. Mild atypical scarlet fever and mitigated measles can hardly be differentiated from the category of atypical exanthems. Anamnestic data often are misleading. The recurrence of the same clinical picture in the same patient, not infrequent in relation to scarlet fever, measles and rubella, is always suggestive of a diagnostic error.

It seemed thus justified to check the clinical diagnosis by virological tests in a number of patients with exanthems hospitalized with various clinical diagnoses. In the period from October, 1965, to November, 1966, 236 patients under 20 years of age were examined virologically. We attempted to isolate virus from throat swabs and faecal samples. From 159 cases paired sera were also tested. In the same period, samples from 222 non-exanthematous control patients of similar age distribution were tested virologically and serologically; from 65 control cases paired sera were also available.

Materials and methods

Patients. In 205 out of the 236 cases the diagnosis at admission was not obvious enough to admit the patient to a special ward. These patients were isolated in boxes. As few as 31 cases were admitted to scarlet fever or measles wards.

For each case four, often inconsistent, diagnoses were available for comparison with one another and with the virological results: (a) diagnosis proposed by the physician who had referred the patient to the hospital; (b) diagnosis at admission; (c) diagnosis established at the ward when the material was submitted to the virological laboratory; (d) diagnosis at discharge. For the purposes of comparison diagnosis (c) seemed the most suitable.

Samples. On the first or second day of hospitalization throat swabs and faecal samples were taken from every patient under study. Throat swabs were washed into PARKER's 199 medium completed with 1% egg white and the usual antibiotics, in a few cases into saline; another throat swab was taken from each case for bacteriological purposes. The samples were prepared as usual and stored at -20°C .

An acute-phase blood sample was taken on the day of admission, a convalescent-phase sample usually 14 to 21 days later, in a few cases as late as 3 to 13 weeks after admission. Sera were inactivated at 56°C for 30 minutes and stored at -20°C .

Cell cultures. Primary monkey-kidney (MK) cell cultures, prepared by YOUNGNER's method [28], were received from the Department of Virology, National Institute of Public Health, Budapest. Primary human embryonic kidney (HK) cell cultures were prepared by essentially the same method. The MK cell line III/1 had been established by RUZICKA [29]. The maintenance fluid was PARKER's 199 with 5% calf serum for the cell lines HeLa and RK13 and serum-free PARKER's 199 for the kidney cell cultures.

Immune sera. The herpes simplex virus antiserum and the enterovirus and adenovirus type sera were kindly supplied by the Department of Virology, National Institute of Public Health, Budapest; the rubella antiserum by DR. J. L. SEVER (Bethesda, Md., USA). The para-influenza and RS rabbit antisera were prepared by us [31]. For the identification of measles and varicella viruses high-titre convalescent sera were used.

Isolation of viruses. Of the samples obtained from exanthematous patients 443, 423, 439, 238 and 150 were inoculated into primary MK cell cultures, MK III/1, RK13, primary HK, and HeLa cell cultures, respectively. Of the control specimens 442 were examined in

both primary MK and MK III/1 cell cultures, 28 also in HeLa cells. The inoculated cultures were incubated at 37° C. without rolling, for 21 to 28 days. Every material was subjected to 2 to 4 serial passages, in most cases after repeated freezing and thawing. If chickenpox was suspected, the sediment of the centrifuged material was used as inoculum. Haemadsorption test with guinea pig erythrocytes was carried out at room temperature at every passage. The supernatants were tested for haemagglutination (HA) with guinea pig and rhesus monkey erythrocytes at room temperature and 37° C, respectively [31, 32].

Identification of isolates was carried out by the neutralization test throughout. Primary MK cell cultures were used in the identification of enteroviruses and measles virus, HeLa cells for adenoviruses and herpes simplex virus, and RK13 cell cultures for rubella virus. To identify *Herpesvirus varicellae* isolates, the principle recommended by TAYLOR-ROBINSON [33] was utilized in Mk III/1 cell cultures.

Serological tests. All paired sera were titrated for antistreptolysin (AST) and for complement-fixing (CF) antibodies to the group-specific adenovirus antigen, a mixture of suspensions of adenovirus types 1-4, 7 and 15. Measles haemagglutination-inhibition (HI) test was prepared from a descendant of the Edmonston strain maintained at the Department of Virology, National Institute of Public Health, Budapest. Human sera were treated with kaolin and adsorbed with the homologous erythrocytes before tested [32, 33]. In the HI test 4 HA units were used. The serum-virus mixtures were kept at 37° C overnight.

Both the CF and HI titrations were performed using TAKÁTSY's [35] Microtitrator technique.

Neutralization tests with human sera. Varicella: a mixture of our *Herpesvirus varicellae* isolates was used in MK III/1 cell cultures. Sera from patients yielding entero- or respiratory viruses were tested against the homologous virus in primary MK and HeLa cells, respectively.

Subsequently to these tests, the sera still in good condition were titrated with the Gilchrist strain of rubella virus in RK13 cell cultures. Prior to this the strain had to be adapted to the RK13 cell line.

Results

(A) Virus isolations and serological results

Eighty-five virus strains were isolated from 468 specimens obtained from 236 exanthematous patients. The distribution of the strains was as follows. Enterovirus: 38, adenovirus: 16, *Herpesvirus varicellae*: 12, measles virus: 11, rubella virus: 4, herpes simplex virus: 2, RS virus: 1, and type 3 parainfluenza virus: 1.

The 442 specimens obtained from the 222 control cases yielded 38 virus strains, viz. 35 enterovirus, 1 adenovirus, 1 herpes simplex virus and mumps virus strains.

The number of strains isolated in different cultures is presented in Table I; this distribution reflects the susceptibility of the different cell cultures.

The isolation rates for the different virus groups and cell cultures are shown in Table II, separately for the exanthematous and control groups, by age distribution.

As to isolation rate, the 6- to 11-month-old children were significantly different from the 1- and 2-year-old ones (47% vs. 14% for the exanthematous group and 38% vs. 6% for the control group). The enterovirus isolation rate was equal in the two groups. *Herpesvirus varicellae* and measles virus were isolated only from exanthematous cases. The two groups could not be compared on the basis of adenovirus excretion, as only 28 control specimens were tested in cultures satisfactorily susceptible to adenoviruses.

Table I
Virus isolation in various cell cultures

		Group of children	HK	MK	III/1	RK13	HeLa
No. of samples tested		E*	238	443	423	439	150
		C*	—	440	350	—	28
Positive	No.	E	18	48	52	35	18
		C	—	35	33	—	2
	%	E	8	11	12	8	1
		C	—	8	9	—	7
Isolates	No.						
Enterovirus	73		3	73	67	—	—
Adenovirus	17		10	—	5	15	17
H. varicellae	12		—	—	12	10	—
Measles virus	11		4	8	—	5	—
Rubella virus	4		—	—	—	4	—
H. hominis	3		1	—	—	1	3
RS and parainfl.	2		—	2	—	—	—
Mumps virus	1		—	—	1	—	—

* E: exanthematous cases

C: control cases.

From 14 exanthematous cases virus was isolated from both throat swab and faeces. From each of two cases two different viruses were isolated. Among the control patients 11 excreted virus both with faeces and throat secretion.

Sera, depending on their condition and available volume, were tested for AST, adenovirus CF antibodies, measles HI antibodies, and neutralizing antibodies to several viruses. The results of these tests are shown in Table III. In the frequency of AST and measles HI antibodies, there were significant differences between the two clinical groups. Neutralization tests were not carried out in virologically negative cases. In positive cases the paired sera were tested against the isolated virus. In this respect the antibody response was at the same level in the two groups. Control sera were not tested for antibodies to measles and rubella virus.

Table II

Isolation rates and distribution of isolates in the tested age groups

	Group of children	Age							Total	
		Months		Years						
		0—5	6—11	1—2	3—5	6—10	11—15	16—20		
No. of children tested	E*	8	34	63	44	50	31	6	236	
	C*	10	21	35	24	57	61	14	222	
Positive	No.	E	1	16	24	13	8	6	1	69
		C	2	3	2	6	7	5	2	27
	%	E	12	47	38	30	16	19	17	29
		C	20	14	6	25	12	7	14	12
Isolates										
Enterovirus	E	1	9	10	7	3	8	—	38	
	C	2	2	2	8	10	9	2	35	
Adenovirus	E	—	6	3	4	3	—	—	16	
	C	—	1	—	—	—	—	—	1	
H. varicellae	E	—	—	6	4	2	—	—	12	
	C	—	—	—	—	—	—	—	—	
Measles virus	E	—	3	4	2	2	—	—	11	
	C	—	—	—	—	—	—	—	—	
Rubella virus	E	—	1	2	—	1	—	—	4	
	C	—	—	—	—	—	—	—	—	
RS and parainfluenza virus	E	—	2	—	—	—	—	—	2	
	C	—	—	—	—	—	—	—	—	
H. hominis	E	—	1	—	—	—	1	—	2	
	C	—	—	—	—	1	—	—	1	
Mumps virus	E	—	—	—	—	—	—	—	—	
	C	—	—	—	—	—	—	1	1	

* E: exanthematous cases.

C: control cases.

(B) The virological results from the clinical aspect

The cases under study were heterogeneous clinically. According to the diagnosis attached to the specimen, the 236 cases could be divided into four categories, *viz.*, scarlet fever: 88 cases (37.3%); measles: 63 cases (26.7%); varicella: 46 cases (19.5%); and atypical exanthems: 39 cases (16.5%). The

Table III
Serological examination of 224 serum pairs

		Group of children	AST	HAI	CF	Neutralization		
				Measles	Adeno	Homologous**	Rubella	H. v.***
No. of serum pairs tested		E	159	158	159	26	80	20
		C	28	28	29	19	—	—
Positive*	No.	E	21	26	13	25	11	17
		C	2	—	2	19	—	—
	%	E	13	16	8	96	14	85
		C	7	—	7	100	—	—

* at least fourfold rise in titre during illness

** homologous entero- and respiratory viruses

*** *H. varicellae*

relative frequencies of these diagnoses for bimonthly periods are presented in Fig. 1.

The relative frequency of cases with scarlet fever diagnosis was 27–50% of the cases examined in the same bimonthly period. It was relatively low in December–January and in August–October. From cases diagnosed as measles the first specimens arrived in the period December–January. The relative frequency of such cases was considerable from February to July, *i.e.*, in the months when the incidence of measles is normally high. Most cases of chicken-pox were examined in December–January. Atypical exanthems constituted 10–15% of all the cases of the year except August–October when such cases amounted to 50% of the 26 cases tested.

In the following each of the four clinical groups is separately compared with the virological and serological results.

1. *Cases indicated as scarlet fever (38 cases).* *Streptococcus haemolyticus* infection was verified bacteriologically in 23.8% of these cases. Viral infection was demonstrated by serological and virological tests in 31.8%. The remaining 44.4% proved negative in all the performed tests. Results are shown in detail in Fig. 2.

Of the 88 cases 6 proved to be measles and another 6 rubella. In 7 cases adenovirus infection (in 2 cases type 2, in 2 cases type 3 adenovirus was isolated, in 3 cases the CF titre rose significantly), in 5 cases echovirus infection (type 3, 4, 8, 11 and 16, respectively), in 5 cases coxsackievirus infection (one case each of A-9, B-1 and B-3, 2 cases of B-5) and in 1 case type 3 parainfluenza virus infection was demonstrated. In 3 cases double infection were present:

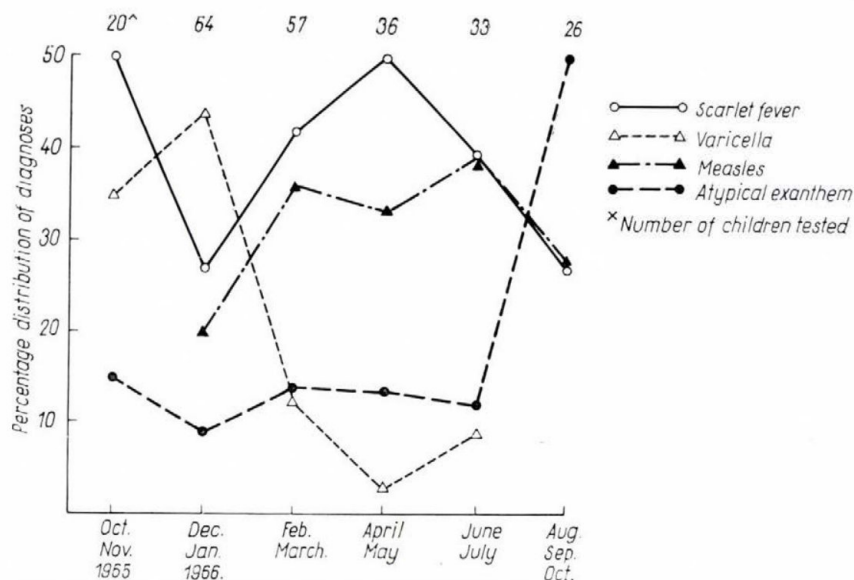


Fig. 1. Seasonal variation of the relative incidence of different clinical diagnoses in the exanthematous group

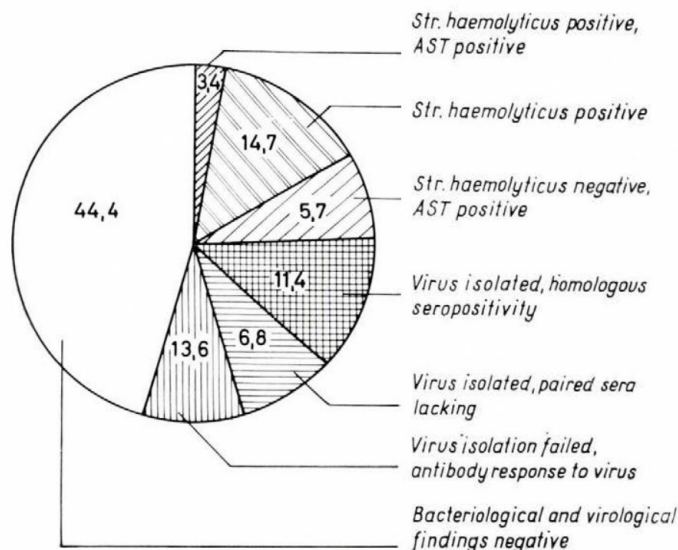


Fig. 2. Clinical diagnosis; scarlet fever (88 cases). Percentage distribution of laboratory findings

adenovirus 2 + echovirus 4, adenovirus 3 + coxsackievirus B-1 virus, rubella virus + rising AST titre, respectively.

2. Cases indicated as measles (64 cases). The clinical diagnosis was verified by virus isolation and/or demonstration of seroconversion in 30.1%. In 44.7%

seroconversion did not take place, whence measles could be excluded. Within the seronegative group infection with other viruses was shown by virus isolation and serological tests in 26.7%. In 25.2% neither paired sera were available nor virus could be isolated (Fig. 3).

An infection other than measles was shown in 24 cases: in 2 cases rubella, in 5 cases adenovirus infection (from 2 cases type 3, from 1 case type 5 virus was isolated, in 2 cases the CF titre indicated the infection), in 3 cases echovirus infection (type 8, 9 and 11, respectively), in 11 cases coxsackievirus infection

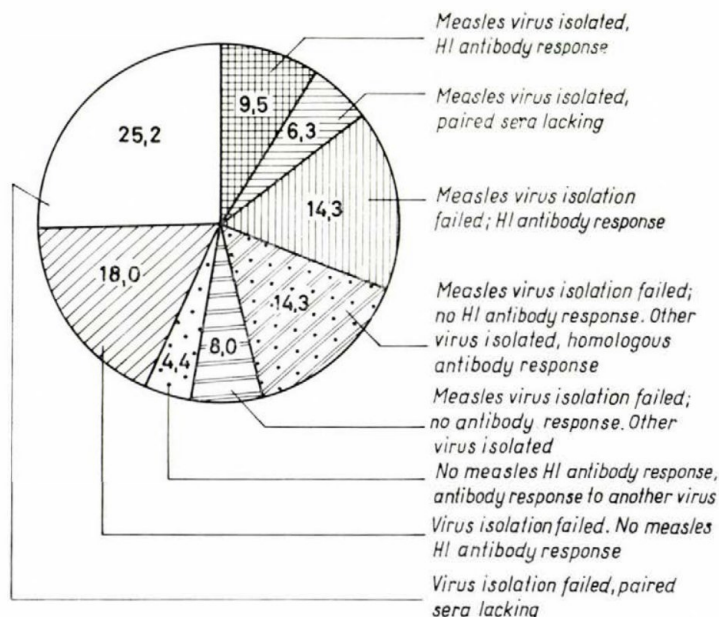


Fig. 3. Clinical diagnosis: measles (63 cases). Percentage distribution of laboratory findings

(in 3, 4, 1 and 3 cases type A-9, B-3, B-4 and B-6, respectively), in 1 case fresh *Str. haemolyticus* infection was demonstrated. From a case of verified measles type 8 echovirus, from another, type 3 adenovirus was isolated.

3. Cases indicated as varicella (46 cases). The diagnosis was verified by virus isolation and/or virus neutralization in 45.7%. In 19.6% some other infection was shown. In the remaining cases neither paired sera were available nor virus was isolated (Fig. 4).

From 4 cases adenovirus was isolated (type 2 from 2 cases, type 5 + type 21 from 1 case, type 6 from 1 case). In further 6 and 5 cases the group-specific adenovirus CF titre showed a two- and fourfold rise, respectively. Varicella + measles double infection was revealed in 2 cases. From a child previously vaccinated with Sabin's type 3 poliovirus vaccine strain this virus was re-isolated.

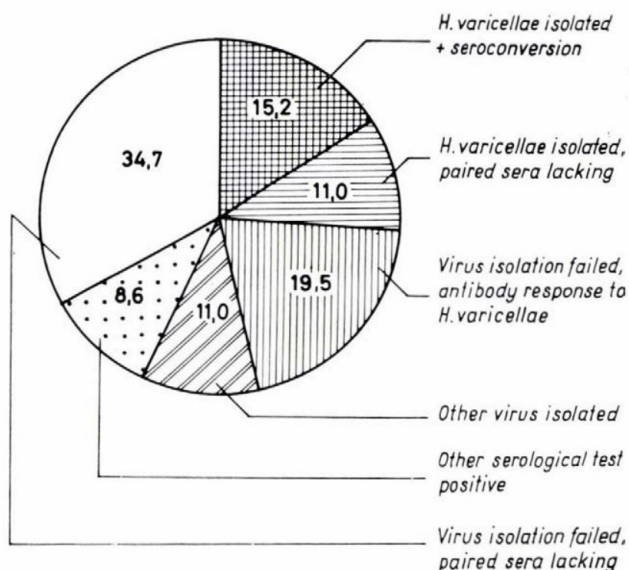


Fig. 4. Clinical diagnosis: varicella (46 cases). Percentage distribution of laboratory findings

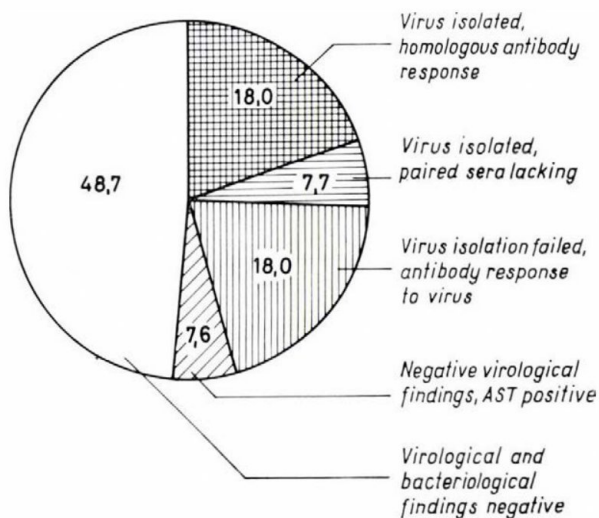


Fig. 5. Clinical diagnosis: atypical exanthem (39 cases). Percentage distribution of laboratory findings

4. Cases indicated as atypical exanthem (34 cases) or rubella (5 cases). Virus infection was demonstrated by virus isolation and/or serological tests in 43.7%. In further 7.6% *Str. haemolyticus* infection was evidenced. Thus the aetiology was made probable in 51.3%. (Fig. 5).

One of the cases proved to be measles, 3 proved to be rubella. In 3 cases adenovirus infection (in 1 case type 5 virus was isolated, in 3 cases the CF titre indicated the infection), in 4 cases coxsackievirus infection (in 3 cases type A-9, in 1 case B-6), in 2 cases herpes simplex virus infection and in one case RS virus infection was present. In 1 case seroconversion took place to both measles and rubella viruses.

The clinical description of the cases will be published elsewhere.

(C) *The control group*

This group could be divided into 5 subgroups; infectious hepatitis, respiratory illness, "viral infection", bacteriologically negative enteritis, and neurological cases.

1. *Infectious hepatitis (87 cases)*. Virus was isolated from 17 cases: type 12 echovirus and coxsackievirus B-1 each from one case and coxsackie A-9 virus from 15 cases. The specimens yielding coxsackie A-9 virus were submitted from the same department in September and October, 1966. In each of these cases seroconversion was demonstrated to the homologous virus. In 2 cases *Str. haemolyticus* infection was present.

2. *Respiratory illness (49 cases)*. Two virus strains were isolated: from one case herpes simplex virus, from the other, a child vaccinated shortly before, type 2 poliovirus. In 1 case the CF test indicated adenovirus infection.

3. The 34 cases indicated as "viral infection" proved to be negative both virologically and serologically.

4. *Enteritis (27 cases)*. Virus was isolated from the faeces in 6 cases (22%): echovirus (types 1, 3, 5 and 16) in 4 cases, type 3 poliovirus in 1 case and type 3 adenovirus in 1 case. Four of the virus excretors were under 1 year of age, the remaining two belonged to the age group from 2 to 4 years.

5. *Neurological cases (25)*. Virus (type 2 echovirus and mumps virus) was isolated from two cases.

Discussion

In the diagnosis of acute exanthematous disease of children clinicians in general do not call in the aid of the virus laboratory, especially not if the course of illness is typical. In atypical cases the diagnosis often remains equivocal. The difficulties in the differential diagnosis may lead to "prestige diagnosis", uncontrollable by virological data until recent times.

The present results, being based on selected cases, do not reflect the general reliability of the clinical diagnosis of exanthematous fevers. Most specimens originated from children isolated in boxes in view of uncertain diagnosis. Nevertheless, the long period of the present study and the considerable number of the cases involved may justify the publication of our unexpected results and allow some conclusions.

The use of cell cultures of different susceptibility spectra made the diagnosis of a great part of marginal cases possible, whereas in some apparently typical cases of measles, scarlet fever and rubella the clinical diagnosis could not be confirmed.

Isolation of measles virus is usually restricted to the prodromal phase and the first one or two days of the exanthematous phase [36]. In several cases we succeeded in isolating measles virus on the 3rd or 4th day after the rash had appeared. In these cases except one the children were seronegative at the time when the positive secretions were taken.

We had to identify measles virus isolates by the time-consuming neutralization test, for 7 of our 11 isolates failed to agglutinate rhesus or vervet monkey erythrocytes and even in the 4 cases when agglutination occurred, the HA titres were too low even after a number of serial passages. The haemadsorption test was also inconsistent.

Taking into account the difficulties encountered in the isolation of measles virus, we emphasize the importance of the examination of paired sera by the HI test, which according to Italian authors [37] is equivalent to, according to ENDERS-RUCKLE [32] more sensitive than, the neutralization test.

HI titres reached their highest value in the third week of illness. Several children had received gamma globulin, but the passive serum antibody never attained the 1 : 20 level and so did not disturb serodiagnosis.

The clinical diagnosis of scarlet fever is almost exclusively based on the cutaneous and mucous membrane manifestations. Isolation of *Str. haemolyticus* is seldom successful — in not more than 20% of the cases — after penicillin treatment has been started. Even serodiagnosis meets with difficulties, since (i) the bacterium is present in the organism for a period too short to induce a measurable antibody response; (ii) AST is specific for streptococcus infection, not for scarlet fever; (iii) there is no practical method to demonstrate the weak antibody response to the erythrogenic toxin. Furthermore, the classical anti-toxic extinction test cannot be performed because of the short duration of the rash. In spite of the difficulties, we have tried to prove the presence of *Str. haemolyticus* infection in our material. The fact that our attempts were successful as rarely as in 23.8% does not speak against the streptococcal aetiology of scarlet fever. Nevertheless, isolation of viruses from 31.8% of the cases diagnosed as scarlet fever deserves attention. Scarlet fever might perhaps be accompanied by an increased susceptibility to viral infection or a scarlatiniform rash may occasionally be caused by viruses. It might be supposed that the virus-induced cases are mild, often atypical, free of complications, and perhaps the viral origin of some scarlatiniform cases might explain the repeated scarlet fever diagnosis in some persons.

The uncertainty of the clinical diagnosis of rubella has been shown by YOUNG and RAMSAY [38], who analysed 400 cases of rubella observed in the

period from 1955 to 1962. Out of our 11 verified rubella cases 6 had been referred to us with the diagnosis of scarlet fever, 2 as measles and 3 as atypical exanthem. In all of these cases except one the diagnosis at discharge was the same. Taking into account the possible occurrence of symptomless rubella, we feel justified in stating that rubella cannot be diagnosed satisfactorily without virological testing.

The inclusion of varicella in the present study appears to be unjustified at first sight, the disease being nearly homogeneous clinically. These cases, however, seemed to be of interest from the virological aspect. *Herpesvirus varicellae* has been isolated from cutaneous eruptions by several authors [33, 39—41]. The frequent occurrence of an enanthem and the respiratory character of varicella infection suggests the presence of the virus in the oropharynx [42]. After several unsuccessful attempts by others, GOLD was [41] the first to isolate the virus from the pharynx; he attributed the previous failures to the poor susceptibility of the tissue cultures (mainly primary human amnion cultures) used.

The III/1 MK cell line was found satisfactorily susceptible to varicella virus by GÉDER *et al.* [43, 44]. For this reason, we employed this cell line in addition to the RK13 cell line. In these cultures we succeeded in isolating the virus from oropharyngeal swabs from 12 cases. All these children had pharyngeal enanthem, or vesicles, most of which opened up on swabbing.

In addition to the classical pathogens, entero- and adenoviruses were often demonstrated in the present study. It is well known that the aetiological role of these viruses often cannot be accepted as a fact even if the virus has been isolated and specific antibody has developed. The isolated virus may be related to a simultaneous symptomless infection. We suppose that the coxsackievirus A-9 infections that occurred in 15 hepatitis cases in September—October, 1966, were symptomless infections, unrelated to the actual illness.

In spite of the virus excretion by some control patients, a causal relationship may be assumed between the enterovirus infection and the actual illness in some of the exanthematous cases.

The following points may be taken into consideration.

(a) The clinical picture of the cases excreting coxsackie- or echovirus was mostly measles-like, but some of the cases with scarlatiniform or atypical rash also excreted these viruses. In the excreters the measles HI test, the AST test and the rubella virus neutralization test were negative.

Group B coxsackieviruses were excreted by exanthematous children in the spring, A-9 strains in the autumn. In each of these cases seroconversion took place to the isolated strain.

(b) In the exanthematous group 70% of the enterovirus excreters were under 5 years of age. Since children under 5 years of age often give a more severe reaction to the same infection than older children do, exanthematous

manifestations may be more frequent among young children. Predisposing factors might also have some role: in two cases a morbilliform condition was preceded by smallpox vaccination.

(c) Coxsackievirus excretion by children with morbilliform, purpuric, maculopapular and urticarious exanthems [2, 7, 11, 12, 16, 17] and echovirus infections of children with morbilliform or scarlatiniform exanthems [1, 4, 12–17] have been reported.

It is much more difficult to evaluate the significance of adenovirus infections and to decide their primary or secondary character. It is of interest that most of the adenovirus infections were associated with dermatitis and allergic skin manifestations. Most of these cases had been referred to us with the diagnosis of scarlet fever.

Adenovirus infection was demonstrated in 9 complicated cases of varicella, and in further 6 such cases a twofold rise in the adenovirus CF titre was shown. As complications, purpuric and erythematous phenomena occurred. It may be assumed that varicella might activate latent adenovirus infections or the actual double infection may have accounted for the complications. The simultaneous occurrence of respiratory symptoms and rash in the course of adenovirus infections has been reported [19–22].

The acute exanthematous diseases of children, just as abacterial meningitis and some respiratory diseases, seem to be clinical but not strict aetiological entities. The role of some enteroviruses appears to be proven, although the pathomechanism of these infections is still unknown. This as well as the supposed role of adenoviruses and, perhaps, of myxoviruses, requires further investigations.

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TAXONOMIC STUDIES ON MYCOBACTERIA ON THE BASIS OF THEIR ANTIGENIC STRUCTURE

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Summary. (i) The antigenic structure of 8 mycobacterial species has been investigated by immune diffusion in homologous and heterologous systems. Common antigenic components have been examined to establish inter-species relationships. The examined organisms were divided into two groups. Group A contained *M. bovis* BCG, *M. kansasii*, *M. avium*, *M. balnei* and *M. simiae*; these organisms contained at least one common antigenic component characteristic of group A. On the basis of specific common antigenic factors *M. phlei* and *M. smegmatis* were classified into group B. *M. fortuitum*, although closely related to *M. smegmatis*, belonged to neither of the above groups.

(ii) In antigenic structure, *M. simiae* n. sp. strain 61 showed the closest relationship to *M. kansasii* and *M. balnei*, but it has more common antigens with saprophytic strains (*M. phlei* and *M. smegmatis*) than any other A group mycobacterium.

(iii) By absorption with heterologous antigens monospecific sera were prepared from the immune sera which reacted practically only with the homologous species. Absorbed sera are suitable for the identification of unknown strains.

In the last two decades studies on mycobacteria isolated from man, animals and environment have been focussed on the taxonomic position of so-called atypical strains. HAUDUROY [1] in 1946 prepared a list of mycobacteria known at that time and concluded that there was no sufficient basis for a classification. BERGEY [2] listed 14 mycobacterial species and classified *M. tuberculosis*, *M. bovis* and *M. microti* as separate species. In reality these organisms are various types of the same species and can be distinguished only by their pathogenicity to different animals. It was necessary, therefore, to review the classification of, and biological relationships between, mycobacteria. RUNYON [3] recommended a practical grouping of atypical mycobacteria on the basis of pigment production and the rate of multiplication. Bacterial classification is based primarily on biochemical activity and antigenic structure. In recent years investigations into these properties yielded promising results. Determination of enzyme activity was found suitable for the differentiation of many pathogenic and saprophytic strains [4]. ADANSON's similarity determination opened up a new way for the classification of mycobacteria [5, 6].

Immunological methods are none the less important. Investigations into the antigenic structure of bacteria allow the demonstration of serological identities, relationships and differences. SCHAEFER [7] elaborated an agglutination method suitable for the identification of several atypical organisms and for the serological subgrouping of biochemically identical strains. Immune diffu-

sion and immune electrophoresis introduced by OUCHTERLONY [8, 9] and GRABAR [10] were applied by PARLETT and YOUNG [11], LIND [12, 13], NORLIN [14], OUCHTERLONY [15], SOUREK and SIR [16], SMOLYANSKAYA [17], TUBOLY [18], GIMPL [19] and WAYNE *et al.* [20]. In previous papers [21, 22, 23] we pointed out that the gel precipitation method was suitable for estimating the degree of relationship between mycobacteria. Thus we showed the close relationship between *M. kansasii* and *M. tuberculosis*. KARASSOVA, WEISZFEILER and KRASNAY [24] isolated from monkeys a new species, *M. simiae*, among a number of mycobacterial strains.

In the present study we examined the antigenic structure in homologous and heterologous precipitation systems for *M. bovis* BCG (representing *M. tuberculosis*), *M. kansasii*, *M. avium*, *M. phlei*, *M. smegmatis*, *M. fortuitum*, *M. balnei* and *M. simiae* strain 61.

Materials and methods

Strains. *M. bovis* BCG 1102; *M. kansasii* 232; *M. kansasii* 12478 (ATCC); *M. avium* Kirchberg; *M. avium* 15768 (ATCC); *M. smegmatis* Bp; *M. smegmatis* 14468 (ATCC); *M. phlei* Bp; *M. phlei* 19249 (ATCC); *M. simiae* 61; *M. fortuitum* 689 (ATCC); *M. balnei* 927 (ATCC).

Immune sera were prepared in sheep and rabbits with strains *M. bovis* BCG 1102, *M. kansasii* 232, *M. simiae* 61; *M. avium* Kirchberg, *M. smegmatis* Bp, and *M. phlei* Bp. Four to 8 week Sauton cultures were filtered, concentrated to one tenth of the original volume and mixed at 1 : 1 proportion with incomplete Freund adjuvant (85% Bayol F + 15% Arlacel A) supplemented with 100 mg/ml homologous bacteria, which had been obtained from the above cultures and treated with 2% phenol for 24 hours. Doses of 0.8 ml and 8 ml antigen were given weekly throughout a 8 week period to rabbits and sheep. Bleeding of the animals was performed 14 days (sheep) and 7 days (rabbits) after the last injection.

Test antigens were prepared by passing the culture through G5 glass filters, concentrating to 1/20–1/100 of the original volume (to 1–2% protein content determined by the biuret reaction) and dialyzing against pH 7.4 veronal buffer. The antigens were preserved with 0.01% Thimerosal.

Absorption experiments were performed with heterologous antigens in doses increasing gradually to an immune serum : antigen proportion of 1 : 1. After adding the antigen, the sera were incubated at 37° C for 2 hours then refrigerated at 4° C for 48 hours and centrifuged. The supernatant was used for further absorptions [25].

Immune diffusion test was carried out by OUCHTERLONY's method modified for matrix technique described by JÓKAY and KARCZAG [26]. The distance between the reservoirs was 8 mm, the thickness of the agar layer was 1.2 mm. The medium consisted of 0.8% Bacto (Difco) agar buffered to pH 7.4 with veronal-HCl and preserved with 0.01% Thimerosal. Incubation lasted for 8 days in a wet chamber at 37° C. After the removal of the matrix, the plates were washed in saline, dried, stained with 0.1% acid fuchsin and photographed.

Results

Table I shows the number of precipitation lines obtained in various homologous and heterologous systems. Precipitation lines in heterologous systems indicate the presence of common antigens. In the homologous systems at least 8 to 15 lines were demonstrated. The optimal reaction with different factors in homologous systems varied with the antigen concentration (Fig. 1). All examined mycobacterial strains contained at least one common component. On the basis of other common antigens the strains were divided into two groups.

Table I

Number of precipitation lines obtained in homologous and heterologous reference systems

Antigens	Antisera								
	Kansasii 232	BCG Paris	Phlei Bp	Smeg- matis Bp	Avium Kirch- berg	Avium ATCC	Simiae 61	For- tuitum ATCC	Balnei ATCC
Kansasii 232	10	5	2	4	3	4	7	2	6
Kansasii 12478	10	5	2	4	3	4	7	2	6
Smegmatis Bp.....	4	1	6	12	2	2	5	4	1
Smegmatis 14468	4	1	6	12	2	2	5	4	1
Phlei Budapest	2	1	12	4	1	2	4	2	1
Phlei 19249	2	1	12	4	1	2	4	2	1
BCG Paris	5	10	1	1	3	3	3	2	2
Avium Kirchberg	3	5	1	2	10	5	5	1	3
Avium ATCC.....	4	3	2	2	5	8	4	1	4
Simiae 61	7	3	4	5	5	4	15	2	4
Fortuitum 689	2	2	2	4	2	2	2	6	2
Balnei 927	6	2	1	1	3	4	4	2	10

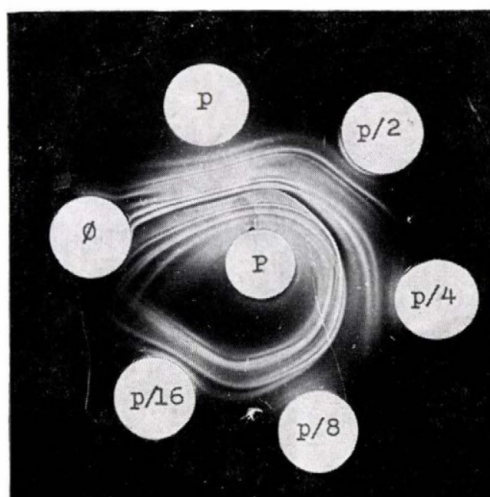


Fig. 1. *M. phlei*-anti-*phlei* homologous precipitation system; antigen dilution, 1 : 2—1 : 16. In Figs 1—9 capitals indicate the immune sera: B = anti-BCG 1102; K = anti-kansasii; A = anti-avium Kirchberg; M = anti-simiae 61; S = anti-smegmatis; P = anti-phlei; Bal = anti-balnei. Antigens are designated with the corresponding small letters. Denominators for the symbols of antigens indicate the degree of dilution. Small letter denominators for sera (e.g. P/ms) mean the antigen(s) used for absorption

There was a marked difference between group A strains (*M. avium*, BCG, *M. kansasii*, *M. simiae* and *M. balnei*) and group B strains (saprophytic *M. phlei* and *M. smegmatis*). *M. fortuitum* contained 4 antigens in common with *M. smegmatis*, but not the antigenic component specific for group B. In group A strains at least one component was present which was not demonstrated in group B strains (Figs 2—4). On the other hand, common antigens character-

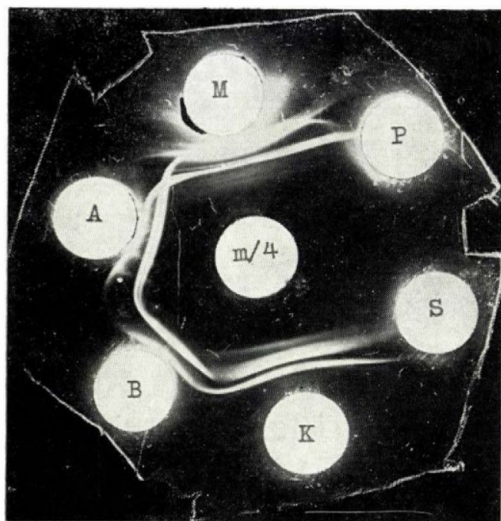


Fig. 2. Central well: *M. simiae* 61 antigen; peripheral wells: reference sera. For explanation see Fig. 1

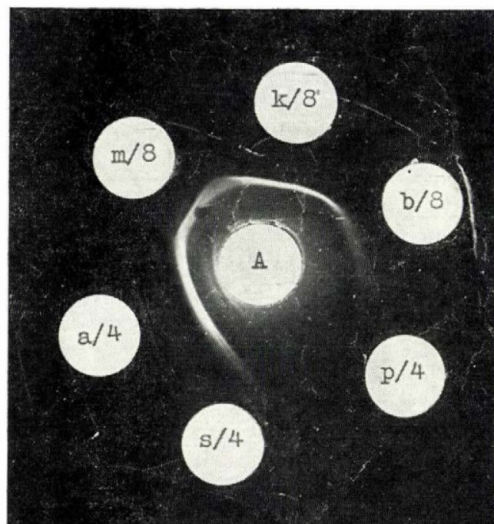


Fig. 3. Central well: anti-avium Kirchberg serum; peripheral wells: reference antigens. For explanation, see Fig. 1

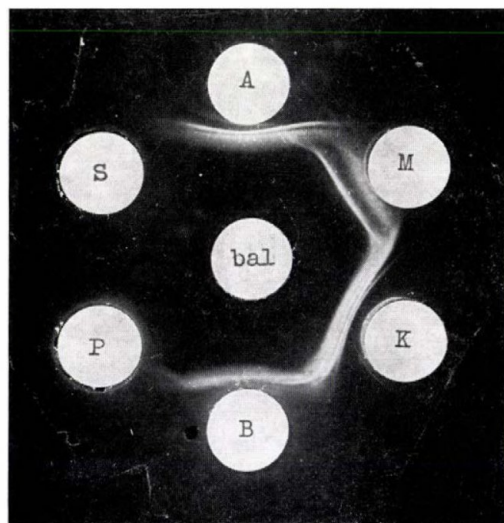


Fig. 4. Central well: *M. balnei* antigen; peripheral wells: reference sera. For explanation, see Fig. 1

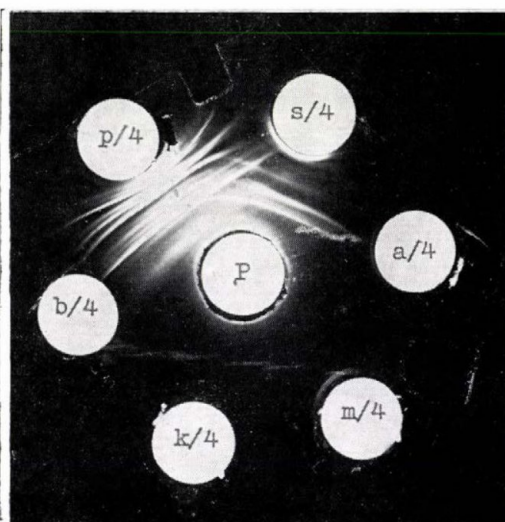


Fig. 5. Central well: anti-phlei serum; peripheral wells: reference antigens. For explanation see Fig. 1

istic of group B strains were absent from *M. avium*, BCG, *M. kansasii*, *M. simiae* and *M. balnei* (Fig. 5). It was demonstrated that *M. phlei*, *M. smegmatis* and *M. kansasii* strains used in this study for immunization were antigenically

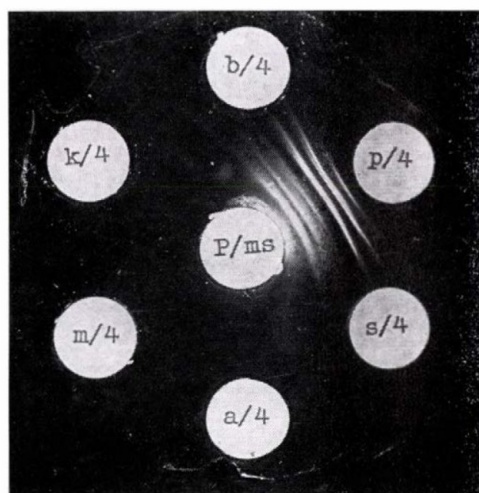


Fig. 6. Central well: anti-phlei serum absorbed by *M. simiae* 61 and *M. smegmatis* (P/ms); peripheral wells: reference antigens. For explanation, see Fig. 1

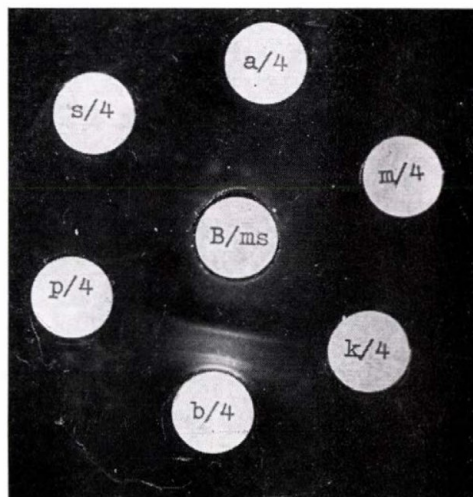


Fig. 7. Central well: anti-BCG 1102 serum absorbed by *M. simiae* 61 and *M. smegmatis* (B/ms); peripheral wells: reference antigens. For explanation, see Fig. 1

Table II

Number of precipitation lines obtained with unabsorbed and absorbed sera in homologous and heterologous reference systems

Antigens	Antisera							
	B	B/ms	K	K/ms	S	S/mp	P	P/ms
BCG Paris	10	5	5	—	1	—	1	—
Kansasii 232	5	—	10	4	4	—	2	—
Kansasii 12478	5	—	10	4	4	—	2	—
Smegmatis Bp.....	1	—	4	1	12	8	6	—
Smegmatis 14468	1	—	4	1	12	8	6	—
Phlei Bp.....	1	—	2	1	4	—	12	6
Phlei 19249	1	—	2	1	4	—	12	6

Abbreviations: B = unabsorbed anti-BCG serum; B/ms = anti-BCG serum absorbed by *M. simiae* 61 and *M. smegmatis* antigens; K = unabsorbed anti-kansasii serum; K/ms = anti-kansasii serum absorbed by *M. simiae* 61 and *M. smegmatis* antigens; S = unabsorbed anti-smegmatis serum; S/mp = anti-smegmatis serum absorbed by *M. simiae* 61 and *M. phlei* antigens; P = unabsorbed anti-phlei serum; P/ms = anti-phlei serum absorbed by *M. simiae* 61 and *M. smegmatis* antigens.

identical with the corresponding international type (ATCC) strains. This finding was confirmed by absorption tests; after absorption with the corresponding ATCC strains, the sera failed to react with the homologous antigens. There was, however, some antigenic difference between *M. avium* Kirchberg and *M. avium* ATCC.

By absorption, "monospecific" sera were prepared; these reacted practically only with the homologous antigen. In order to prepare such sera, the immune sera had to be absorbed with one A and one B group strain. Among A group strains, *M. simiae* 61 was considered best for this purpose, as it contained

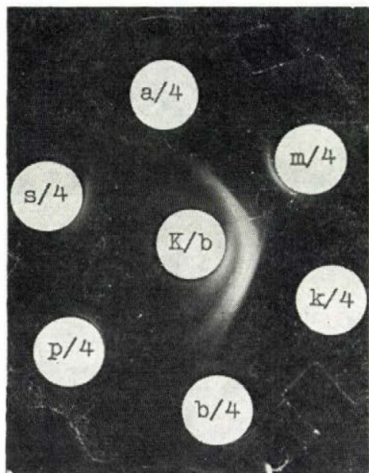


Fig. 8. Central well: anti-kansasii serum absorbed by BCG (K/b); peripheral wells: reference antigens. For explanation, see Fig. 1

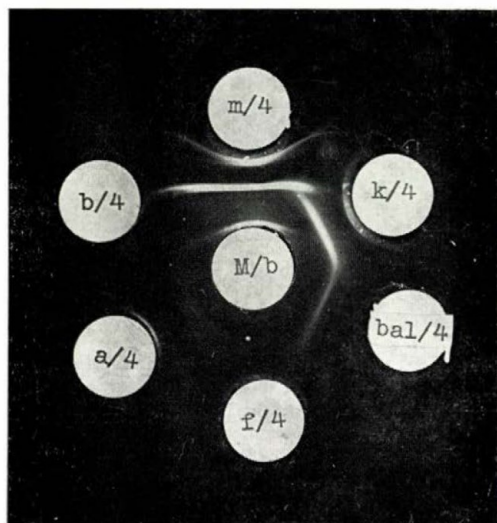


Fig. 9. Central well: anti-simiae 61 serum absorbed by BCG (M/b); peripheral wells: reference antigens. For explanation, see Fig. 1

the largest number of common antigenic components. Anti-BCG, anti-kansasii, anti-avium and anti-phlei sera were absorbed in addition to *M. simiae* with *M. smegmatis* (Table II and Figs 6, 7). The anti-simiae serum was absorbed with *M. kansasii* and *M. smegmatis* antigens and the anti-smegmatis serum with *M. phlei* and *M. simiae* antigens.

The presence of numerous common antigens and the result of absorption tests indicated that within group A *M. kansasii*, *M. simiae* and *M. balnei* are closely related. The immune serum prepared against *M. kansasii* ceased to react with *M. avium* after absorption with the BCG strain, although it still contained antibodies for identical or related components of *M. simiae* (Figs 8, 9). *M. kansasii*, *M. simiae* and *M. balnei* contain, therefore, common components which are absent from avium and BCG antigens.

Discussion

Serological methods are successfully applied for the classification and identification of bacteria. OUCHTERLONY's immune diffusion method has proved advantageous for the study of mycobacterial antigens. The technique applied

SATURATED FATTY ACIDS IN POLIOVIRUS HOST CELL INTERACTION

I. STIMULATION AND INHIBITION OF VIRION UPTAKE

By

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Summary. Saturated fatty acids of C_{16} to C_{24} even chain lengths were studied in respect of their effect on uptake of adsorbed poliovirus by cells of a permanent monkey kidney cell line. The effect was referred to that of 3×10^{-5} M bovine albumin as 100.

Dose response curves taken with different fatty acids have shown that up to C_{18} chain length (stearic acid) the effect does not exceed 50% at any concentration. C_{24} chain length fatty acid (lignoceric acid) failed to exert any effect in the concentration ranges tested. Arachidic (C_{20}) and behenic (C_{22}) acids were of about 90% activity at 10^{-5} M and 10^{-7} M concentrations, respectively. With both substances, dose response curves were bell-shaped, suggesting an agonist to antagonist transition of their effects with increasing concentration.

Virions incubated at 37° C for 3 hours with any of the fatty acids tested maintained unimpaired infectivity.

Incubation of cells with any fatty acid tested for 1 hour at 37° C rendered them unable to adsorb virions in a fatty acid free medium. This effect seemed to decrease on prolonged incubation (above 30 minutes) of cells in the absence of fatty acids.

A possible explanation of the mechanism of fatty acid action is offered.

In some previous studies it has been shown that the presence of bovine albumin (BA) or of an appropriate fatty acid stimulates uptake by the host cell of adsorbed virions [1, 2, 3]. The present paper is a report on further details concerning that phenomenon.

Materials and methods

The virus (polio type 1, Mahoney), the permanent monkey kidney cell line (PMK III/1), as well as the methods of maintenance, one-step growth experiments and plaque assay were the same as described previously [1].

Treatment of cells with fatty acid. In 10 ml total volume 10^7 suspended cells were incubated for 1 hour in the presence of the required molar concentration of the given fatty acid. The system was incubated in 5% CO_2 atmosphere under constant gentle shaking in a 37° C water bath. After incubation, the system was centrifuged and the cells were resuspended in a fatty acid-free medium for further study.

Assay of virus adsorption. Cells grown in 2 litre Roux flasks were suspended by versenization and washed twice in HANKS' balanced salt solution (HBS) by centrifugation. The last sediment was suspended to obtain 10^7 cells per ml. One ml of this suspension was added to 3.5 ml of an appropriate medium in a 50 ml siliconed airtight flask. Subsequently, 0.5 ml of an appropriate dilution of stock virus to give input multiplicity 0.1 was added to the system. Incubation took place in 5% CO_2 atmosphere under continuous gentle shaking in a 37° C water bath. Withdrawal of samples for titration of unbound virus in the supernatant was followed by readjustment of the atmosphere's CO_2 content.

Samples for virus assay were immediately diluted 1 : 10 in ice cold HBS and centrifuged for 5 minutes at 1500 rpm. The supernatants obtained were titrated either immediately or within a week after storage at -20° C.

Chemicals. All chemicals used were the same as specified in a previous paper [3].

Behenic and lignoceric acids were kindly supplied by DR. M. KRÁMER from the analytical standards of the National Institute of Nutrition, Budapest.

Fatty acid solutions were prepared freshly in boiling absolute ethanol and diluted in ethanol at 37° C. Final dilutions in the 37° C aqueous systems were prepared with siliconed glassware in a single 1 : 100 step from the appropriate dilutions in warm ethanol.

With this method, well reproducible results were obtained. With aged ethanol solutions, reproducibility was poor; it improved, however, on re-boiling the solution.

Experimental

Effect of fatty acids on the virions. Stock virion suspensions of 5×10^7 PFU/ml were mixed each with equal amounts of 10^{-5} M to 10^{-9} M fatty acids in HBS. Incubation was done under gentle stirring for 3 hours at 37° C followed by a serial dilution of the virions in HBS + 3×10^{-5} BA (HBS + BA) and titration. Neither decrease, nor increase of infectivity was observed on comparing the geometric mean titres obtained in 5 parallel titrations each. In another experiment, a stock virion suspension was diluted serially up to 10^{-7} . To various concentrations of the different fatty acids in HBS, equal amounts of 10^{-6} or 10^{-7} dilutions of the virions were added. After incubation for 3 hours at 37° C, the virion content of the mixtures was determined by plaque assay. Within the limits of the method's error, no changes were detectable in the plaque count in either system as compared to controls in HBS + BA.

The infectivity of virions was found, by both methods, to be unaffected by incubation in the presence of any fatty acid tested at any concentration.

Determination of dose response curves with fatty acids. The infection enhancing effect of the even series of saturated fatty acids of C_{16} to C_{24} chain length was studied. Each acid was tested within the concentration range of 10^{-4} (10^{-5}) M to 10^{-8} M. The complete set of dilutions of the fatty acid under test was examined simultaneously in one and the same experiment to ensure uniformity of the system. Each experiment was repeated at least 3 times. Cells were brought into contact with fatty acids only after adsorption of virus at an input multiplicity 1 for 10 minutes at 22° C and for an additional 5 minutes at 37° C. Unbound virus was removed by washing. Appropriate controls in HBS and in HBS + BA were set up with each experiment. The one step experiments were performed as described in a previous paper [1]. Results are shown in Fig. 1.

Short chain (C_{16} and C_{18}) fatty acids exhibited not more than about 50% activity even at the highest concentrations available for testing in HBS. Arachidic (C_{20}) and behenic (C_{22}) acids exhibited about 90 to 95% activity at 10^{-5} and 10^{-7} M concentration, respectively. Activity of both acids declined rapidly on lowering or increasing their concentration. Lignoceric acid (C_{24}) was inactive throughout the concentration range tested (10^{-5} M to 10^{-9} M).

in the present investigation renders the method more sensitive and makes a refilling of the reservoirs unnecessary. Although the determination of the number of precipitation lines may give some orientation about the identity of, or difference in, the antigens, it does not always reflect the degree of relationship. The presence or absence of common antigenic components may serve as a basis for estimating the degree of relationship. LIND pointed out that although the method was advantageous in many respects, standardization of antigen and immune serum presented difficulties arising in almost all phases of the test from precipitation reaction to evaluation.

It would appear that for serological classification homologous reference systems containing the largest possible number of components representing a wide variety of species, group and generic antigenic patterns are needed. While the presence of large numbers of antigenic components in the homologous reference systems provides a better chance for including the strains into various subgroups, identification of a single factor in such systems is obviously difficult. Thus, reference systems with relatively few but characteristic antigens are preferable. For immunochemical (absorption) studies it seems advantageous to use several reference systems for the preparation of species, group, etc. specific sera. Our "monospecific" sera should be considered as result of preliminary experiments. Repeated absorption seemed to yield better results than a single absorption, although it failed to remove all cross-reacting components from anti-simiae 61 and anti-kansasii sera. Application of such relatively monospecific sera facilitates the identification of unknown strains. These sera are relatively specific for the corresponding mycobacterial species, as they react weakly or not at all with the common group and generic antigens. This consideration is of course valid only for the examined species. Other mycobacterial species may give partial reactions with our "monospecific" sera. These absorbed sera are useful primarily in the identification of unknown strains. In serological classification, the detection of common antigenic components characteristic of smaller or larger mycobacterial groups is more important. Division of our strains into groups A and B was performed on the basis of characteristic group-specific antigenic components.

The antigenic component characterizing group A strains is present in pathogenic (*M. kansasii*, BCG, *M. avium*, *M. balnei*) and facultatively pathogenic (*M. simiae*) mycobacteria. Group B antigen was demonstrated in saprophytic organisms (*M. phlei*, *M. smegmatis*). *M. fortuitum* is more closely related to *M. smegmatis* than to *M. phlei*; whether or not it can be included in group B awaits further studies. Within group A, *M. simiae* n. sp. strain 61 stands close to *M. kansasii* and *M. balnei* as these strains contain common components that are absent from the other examined mycobacteria. On the basis of these common antigens, group A can be divided into subgroups. *M. simiae* strain 61 cannot be regarded as the typical representative of *M. simiae*

until a serological study in progress has compared it with other strains isolated from monkeys.

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It should be noted that the activity of BA also appeared to decrease rapidly at higher as well as at lower concentrations.

These effects having been observed in systems containing virions already adsorbed to the cells, it seemed to be of interest to test cells treated with fatty acids prior to virion adsorption.

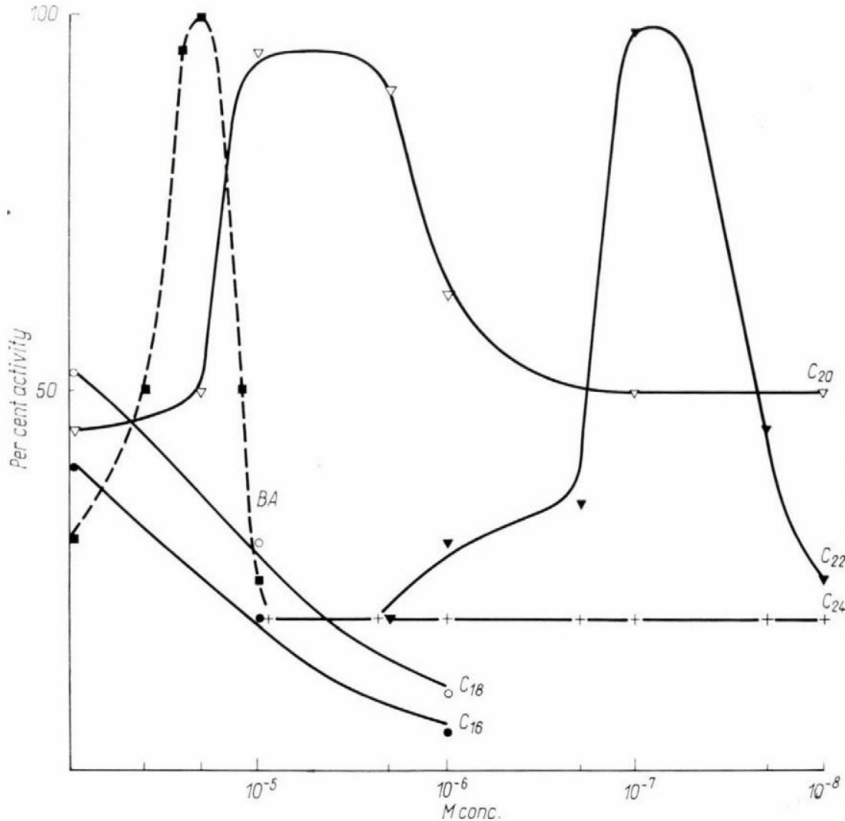


Fig. 1. Dose-response curves of fatty acid effect on virion uptake. BA = bovine albumin: C_{16} = palmitic acid; C_{18} = stearic acid; C_{20} = arachidic acid; C_{22} = behenic acid; C_{24} = lignoceric acid

Pretreatment of cells with fatty acids. Cells were pretreated with palmitic (C_{16}) or arachidic (C_{20}) acid. To the suspended washed cells virions were added at input multiplicity 1. Adsorption was allowed to take place as described above. Unbound virions were removed by two washings in HBS. From the sediment, 10^6 cells in 1 ml were transferred to 100 ml flasks containing 9 ml HBS. Cells pretreated with HBS or with HBS + BA were included as controls. These systems were incubated under gentle shaking in a water bath at 37°C for 6 hours and the final yield was determined. Results are presented in Table I. Yields obtained in systems with fatty acids added after the adsorption of

Table I

Effect of fatty acids on per cent final virus yield

Substance in HANKS'		Time of treatment relative to virus adsorption		
Nature	Conc. (M)	A 1 hour before	B 0—1 hour after	B/A
0	0	9.00	9.00	1
Bovine albumin	3×10^{-5}	100.00	100.00	1
Arachidic acid	10^{-4}	0.00	47.00	—
	10^{-5}	0.12	90.00	750
	10^{-6}	0.27	57.00	211
	10^{-7}	1.80	50.00	27
	10^{-8}	1.60	50.00	31
	10^{-9}	1.80	47.00	26
Palmitic acid	10^{-4}	0.00	36.00	—
	10^{-5}	0.00	26.00	—

virions are also shown for comparison's sake. Each figure represents the geometric mean of at least 3 independent sets of determinations.

Incubation of cells in HBS or in HBS + BA prior to the addition of virions did not affect the final yields obtained in the respective controls. Pretreatment with arachidic or palmitic acid, however, was found to elicit an about 25 to 750 fold reduction of virus production as compared to that obtained with an identical fatty acid concentration added within 1 hour following virion adsorption.

Arachidic acid and virion adsorption. The phenomenon observed suggested that pretreatment of the cells with a fatty acid may interfere either with adsorption or with uptake of virions. Inhibition of intracellular virus replication seems to be less probable in the light of our previous results [1, 3]. Inhibition of adsorption was studied first. The experiment was performed as described above. Each individual point of the curves shown in Fig. 2 represents the geometric mean of at least 3 determinations.

Pretreatment of cells with 10^{-5} or 10^{-7} M arachidic acid for 1 hour at 37° C resulted in complete inhibition of virus adsorption during the first 15 minutes. The inhibitory effect disappeared on prolonged exposure and adsorption started after an about 30-minute lag. In the controls adsorption started immediately and was similar in rate in both HBS and HBS + BA during the first 15 minutes (20 and 30%). Subsequently the rate of adsorption decreased considerably (by about 50%) in plain HBS as compared to HBS + BA.

This was, however, a test involving considerable sources of error particularly when the amount of virus adsorbed was less than 25%. With a one

step growth curve experiment, the infection of a single cell should theoretically be demonstrable, provided it shows the normal 10^3 final virus yield. Therefore, the effect on the yield was studied of fatty acid added simultaneously with the

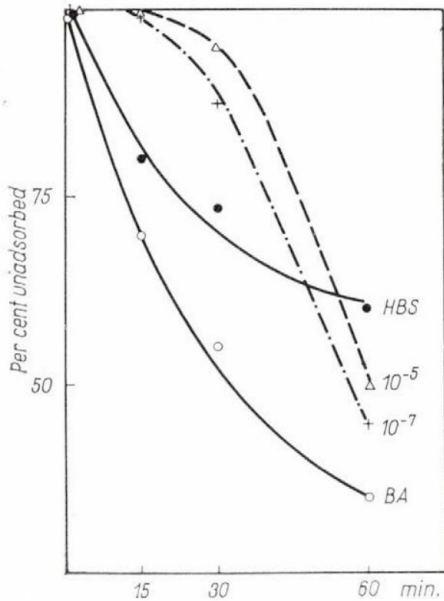


Fig. 2. Inhibition of virion adsorption by arachidic acid pretreatment of the cells. BA = bovine albumin; HBS = Hanks' balanced salt solution; 10^{-5} and 10^{-7} = molar concentrations of arachidic acid

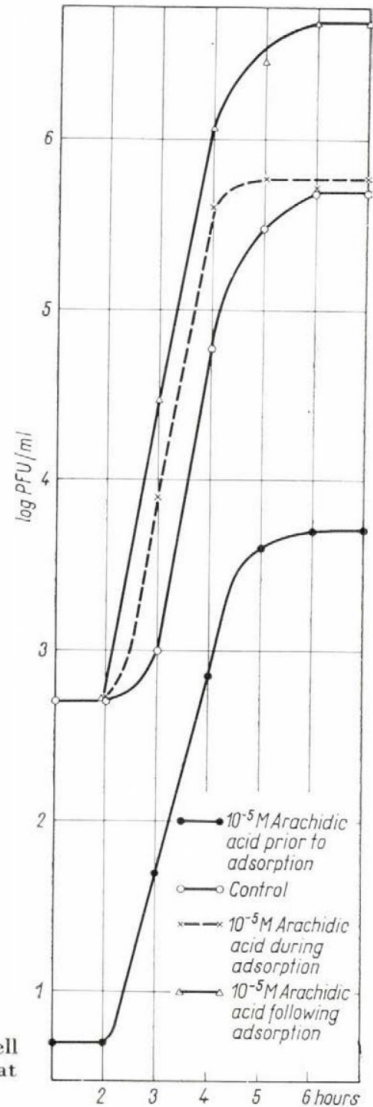


Fig. 3. One step growth curves in host cell virion systems treated with fatty acid at different points of time

virions and allowed to adsorb for 10 minutes at 22°C and an additional 5 minutes at 37°C . Appropriate controls were included as shown in Fig. 3.

These curves revealed two facts. First, the presence of the fatty acid invariably resulted in a reduction of the lag phase. Second, the number of successfully infected cells varied within the range of 3 log units, depending

on the time of addition of fatty acid relative to virus adsorption. As compared to the yield in the absence of fatty acid, a 10fold elevation and a 100fold reduction occurred if the fatty acid was added after and prior to virus adsorption, respectively. The lag phase was reduced in both cases by about 30 minutes. The simultaneous presence of fatty acid and virions during adsorption neither reduced nor increased the final yield relative to that in HBS, whilst it caused an about 10-minute shortening of the lag phase.

Discussion

A previous study from this laboratory has already suggested the decisive importance of three factors in the mechanism of fatty acid action on the initial phase of poliovirus host cell interaction, *viz.* chain length, concentration and time of addition. The results presented here are discussed in this sequence.

In his survey on the structure of cell membranes WILLMER [4] has stated that "certain general properties are common to large groups of such membranes. In one such group it is likely that close packing of long chain (C_{16} to C_{22}) hydrocarbons form the essential feature". HAYDON and TAYLOR [5] found that models of bimolecular phospholipid leaflets would readily accommodate quantities of fatty acids of chain lengths close to that of the membrane's hydrocarbon layer without loss of stability, but with a considerable increase of surface areas. The "wedge" argument valid for the short chain molecules did not appear directly applicable to long chain ones. The said authors stated that with fatty acids of structures not fitting with the leaflet lipids "either the adsorption will be modified by a factor due to exposure of hydrocarbon to water in the final structure, or the leaflet will distort as in the case of short chain molecules".

Considering our results concerning the chain length and optimum viral infection enhancing effect of fatty acids, we have arrived at the conclusion that arachidic and behenic acids (C_{20} and C_{22}) appear to be those best fitting with the hydrocarbons of the membrane of PMK III/1 cells. The shorter and the longer molecules tested were apparently distorting the membrane structure in a way unfavourable for the enhancement of the adsorbed virion's uptake.

The bell-shaped dose-response curves obtained with the two active fatty acids and BA formally resembled those discussed by ARIENS [6] in context of the interaction of two drugs with multiple action having two separate but mutually interacting receptors. The analogy is particularly obvious for the case where one of the two drugs exhibits a transition from increasing agonistic into increasing antagonistic activity parallel to the elevation of its concentration. Although the experimental results do not contradict this possibility, too

little is known about the system examined to justify any direct application of the formulas proposed by ARIENS.

To analyze the effect of the time of addition, let us consider the system used in the present study at 0 time of a typical one step growth experiment. A given number of virions are adsorbed as a result of random collisions with certain properly active and adequately patternized sites of the cell membrane. Such sites are designated as receptor sites. It has been shown [7, 8] that only a small proportion of adsorbed virions will penetrate the cell and initiate a reproductive cycle. Should this happen, it is a consequence of a series of events triggered by an adequate virion-host cell interaction governed by a certain sequence of favourable chance coincidences. We have suggested the possibility [9] that one of the factors involved might be the actual number of virion vertex and receptor site contacts. Optimum attachment has been supposed to take place if the three vertices on one and the same facet attached to three receptor sites. This was assumed to happen to some virions adsorbed to cells maintained in plain HBS. Another part of virions will, however, lack full chance but apparently remain for about 30 to 60 minutes in a metastable state which still allows for its being taken up with some "help". These would be the virions attached through one or two adjacent vertices to one or two receptor sites ("suboptimal attachment"). Addition of the proper fatty acid at adequate concentration at a definite point of time to the system in HBS would represent the "help" required. Initially, we supposed the fatty acids to play some "anchoring" role. Results of the present experiments have necessitated a revision of that assumption. Information available on the effect of fatty acids on the cell membrane's structure as well as the obviously demonstrable rigorous requirement for chain length and concentration rather suggest that the activity of fatty acids involves a certain orienting effect on the membrane's charge pattern in the vicinity of the suboptimally attached virion. The lower or the higher the concentration, and the farther the time of addition from the optimal, the poorer will be the effect.

Pretreatment of the cells with fatty acid inhibited the adsorption of virions, but this inhibition decreased with time provided fatty acids were removed from the bulk phase. Thus it seems that cells would take up fatty acids as well as virions, both inducing appropriate alteration of the cell membrane's structure. The nature of these alterations seems, however, to be different. Uptake of fatty acids stimulates the cell's pinocytotic and phagocytotic activities. This phenomenon does not appear to be specific in respect of definite requirements of chain length, concentration and time of addition [9, 10]. Moreover there is evidence available that once phagocytosis is triggered by a given stimulus, the involved area or the total cell membrane will become refractory to further stimulation for a certain time [11]. This would explain the dramatic inhibition of virion uptake by fatty acid pretreated cells. Recovery

of normal sensitivity takes about 30 to 60 minutes as shown by the adsorption experiment in Fig. 2, and also by some preliminary one step growth studies to be published later.

In contrast to the stimulatory effect, inhibition of virion adsorption did not appear to depend on chain length provided the cells were exposed to fatty acid first and to the virion one hour later. Under similar conditions, variation of fatty acid concentration affected the grade of inhibition only moderately (from 98.2 to 100%), whereas that of stimulation very efficiently (from 9 to 100%). Thus the conditions of inhibition of virion adsorption seemed to resemble those of stimulation of phagocytosis [10]. This similarity suggests that certain non-specific reversible alterations of the cell membrane's structure take place on treatment with any even saturated fatty acid at any concentration tested. The alterations are maintained for about 30 to 60 minutes following the removal of fatty acid from the bulk phase as shown by both virus adsorption and phagocytosis stimulation experiments. The length of this period is quite similar to that required by the cells to metabolize fatty acids adsorbed to their surfaces [12] and to that available for stimulating uptake of adsorbed virions by addition of fatty acids [3].

The direct effect of certain fatty acids on the receptor is suggested by the experiments performed by DR. M. SIMON in this institute [13]. He found that fatty acid treatment of a purified receptor preparation abolished its specific haemagglutination inhibitory effect on ECHO 7 virus, while it had no effect on the virion itself or on the red blood cells themselves. The virion-receptor complex once formed was insusceptible to the effect of fatty acids.

It is supposed that the attachment of a virion to appropriate receptor site(s) induces certain changes in the membrane structure. Suboptimal attachment seems transitorily to sensitize the surface to the stimulatory effect of an appropriate fatty acid present at adequate concentration at the moment required. Delayed addition of the stimulant fails to affect uptake.

BA appeared to exert its favourable influence on virion uptake independently of the time of its addition. BA has been shown to carry several molecules of adsorbed fatty acids which are progressively released on reaction with sites having greater affinities to fatty acids than has BA. Such sites on the cell membrane are thought to develop following suboptimal interaction with a virion and to result in a properly timed exchange of bound fatty acids between BA and the cell membrane.

The above considerations imply the necessity of replacing by some more dynamic model the mechanistic static model of the structure and function of receptors and receptor sites offered in our preliminary working hypothesis [3].

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SATURATED FATTY ACIDS IN POLIOVIRUS HOST CELL INTERACTION

II. MODEL EXPERIMENT ON STIMULATION OF PHAGOCYTOSIS

By

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Summary. Cells of a permanent monkey kidney line (PMK III/1) engulfed considerable amounts of Dowex granules if stimulated by the addition of 3×10^{-5} M bovine albumin fraction V or of 10^{-9} to 10^{-5} M arachidic or palmitic acid. Unstimulated cells engulfed Dowex granules poorly. Treatment with fatty acids rendered the cytoplasm clearer and more homogeneous. Bovine albumin elicited the formation of some large vacuoles at the borders of cells along the free margins of the monolayer.

Stimulation of phagocytosis of Dowex beads by the substances tested appeared to be independent of their concentration within the range of 10^{-9} to 10^{-5} M and of the time of their addition as well.

The mechanism of phagocytosis stimulation by these substances appeared to be different from that involved in their infection enhancing effect in a poliovirus — host cell system.

Several substances including certain essential fatty acids have recently been reported to stimulate pinocytotic activity of cultured mammalian cells [1]. Long chain saturated fatty acids have been shown to enhance *in vitro* the infection by poliovirus of the permanent monkey kidney cell line PMK III/1 [2]. It seemed therefore interesting to examine whether or not the mechanisms involved in the two phenomena were similar.

WEISMAN and KORN [3] used latex beads to study the conditions and kinetics of phagocytosis in *Acanthamoeba*. This method appeared to offer a relatively simple means for the semiquantitative estimation of cell membrane activation in the system used in the present study.

Materials and methods

Cells. Five days old monolayer tube cultures were used of the permanent monkey kidney cell line PMK III/1 isolated by RUZICKA [4]. The cells were grown in a medium consisting of 54% HANKS' balanced salt solution (HBS), 40% PARKER's 199 medium 0.5% bovine albumin, 5% calf serum and 0.5% lactalbumin hydrolysate. Tubes were inoculated with 2×10^5 cells each in 1 ml volume and incubated at 37° C.

Bovine albumin. (BA) This was a Cohn fraction V produced by Browning Chemical Corp., New York. A 10% stock solution was prepared in distilled water, sterilized by filtration and diluted as required in HBS. The molecular weight of BA was taken as 60,000.

Fatty acids. Palmitic acid was kindly supplied by DR. M. KRÁMER (Institute of Nutrition, Budapest). Arachidic acid was a product of Fluka A. G., Switzerland. Stock solutions and final dilutions were prepared freshly for each experiment as described earlier [2].

Dowex beads. Dow polystyrene Latex of Dow Chemical Co. Mich. was used. Granule diameter was 0.264 μ . An 1 : 4,000 final dilution in pH 7.2 HBS of a 3.5% weight stock solution was used.

Histological procedures. Monolayers were fixed *in situ* for 10 minutes with a 10% solution of neutral formaline in pH 7.2 phosphate buffer. They were removed *in toto* using the SZENT-IVÁNYI [5] modification of the ENDERS and PEEBLES method [6]. Slide preparations were stained by MAYER's acid-haemalaun eosin, dried, cleared up in a phenol-xylol mixture and covered.

Experimental

The cultures of PMK III/1 grown for 5 days at 37° C were used. Prior to the experiment cultures were thoroughly rinsed with HBS. The experiments were performed at 37° C in a water bath. All ingredients were preheated to this

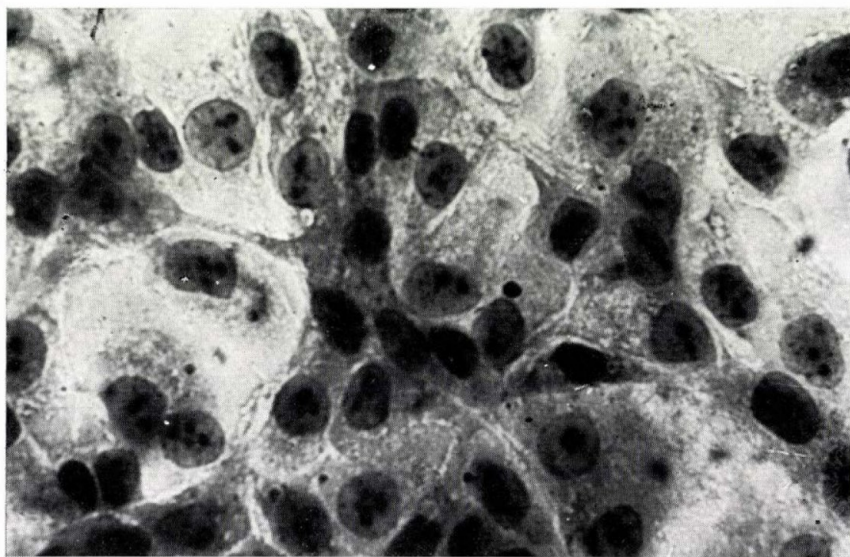


Fig. 1. Cells incubated in HBS for 90 minutes at 37° C. Cell margins clearly distinguishable cytoplasm finely granulated, no conspicuous vacuolization (negative)

temperature. Ten ml experimental medium was used per tube to ensure full submersion of the monolayer. Sedimentation of the Dowex beads was prevented by constant gentle shaking. Every experiment was performed three times on three different occasions using 3 to 5 parallel sets of tubes each.

Our aim was to detect whether the phagocytosis stimulating effect of fatty acids depended on factors similar to those involved in their infection enhancing activity in a poliovirus-host cell system studied in this laboratory [2]. A precise photometric analysis of the amount of beads ingested per cell [3] appeared unnecessary as a semiquantitative estimation based on the microscopic appearance at a magnification of $\times 280$ proved to yield reliable results. The grade of stimulation was evaluated in terms of the incidence of vacuolized cells per field and the number of readily distinguishable vacuoles per cell. Figs 1–5 and the relevant legends give full account of the principles of evalu-

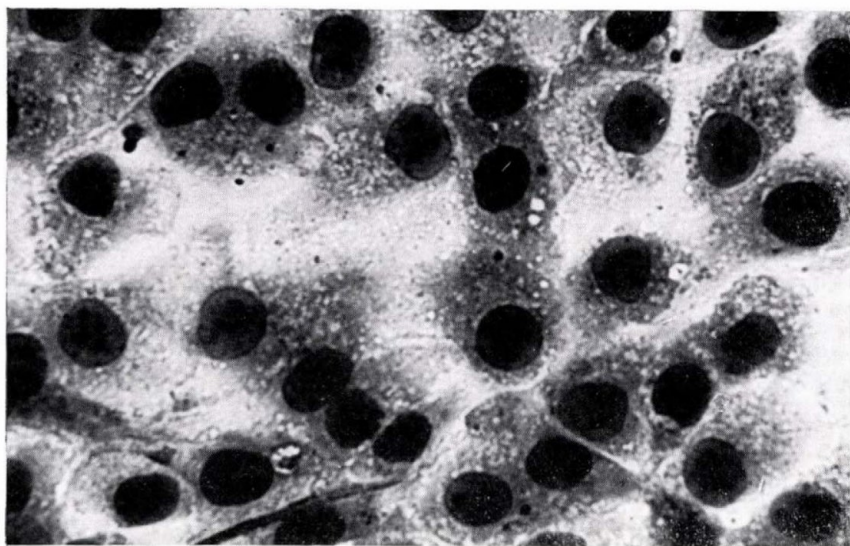


Fig. 2. Cells incubated in HBS + Dowex beads for 30 minutes at 37° C. Cell margins distinguishable, cytoplasm finely granulated, numerous minute vacuoles. One to three conspicuous vacuoles are present in 1 to 3 cells per 50 cell fields (1+)

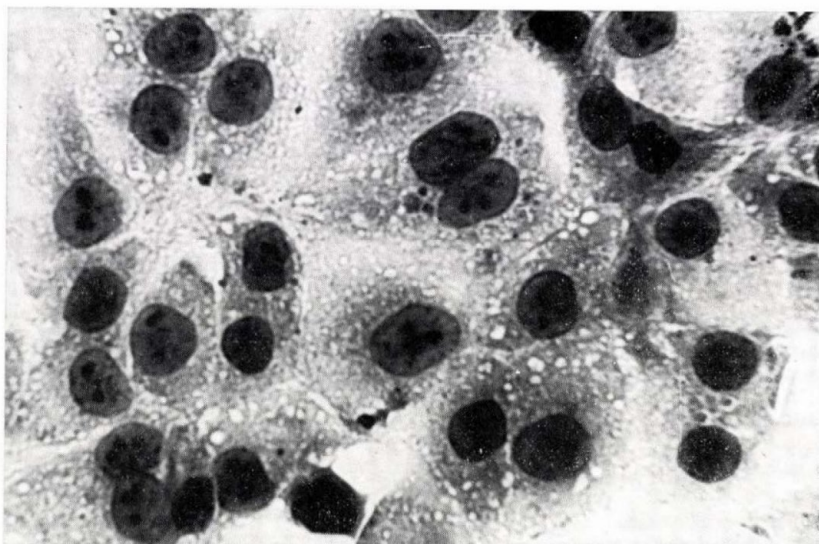


Fig. 3. Cells incubated in HBS + Dowex beads for 90 minutes at 37° C. Cell margins distinguishable, cytoplasm clear with slightly foamy structure. Six to ten conspicuous vacuoles are present in 10 to 15 cells per 50 cell fields (2+)

ation. Photographs of Figs 1—5 and Figs 6—10 were taken under identical conditions at $\times 280$ and at $\times 1000$ direct magnification, respectively.

As a set of controls, monolayers were incubated for 90 minutes at 37°C in HBS, in HBS plus $3 \times 10^{-5}\text{ M}$ BA or in HBS plus 10^{-9} to 10^{-5} M arachidic or palmitic acid, or in HBS with Dowex granules.

No vacuolization was induced by either fatty acid at either concentration. As compared to the controls (Fig. 6) the cells exhibited a moderate reduction of cytoplasmic granulation and staining (Fig. 7). Cells incubated with

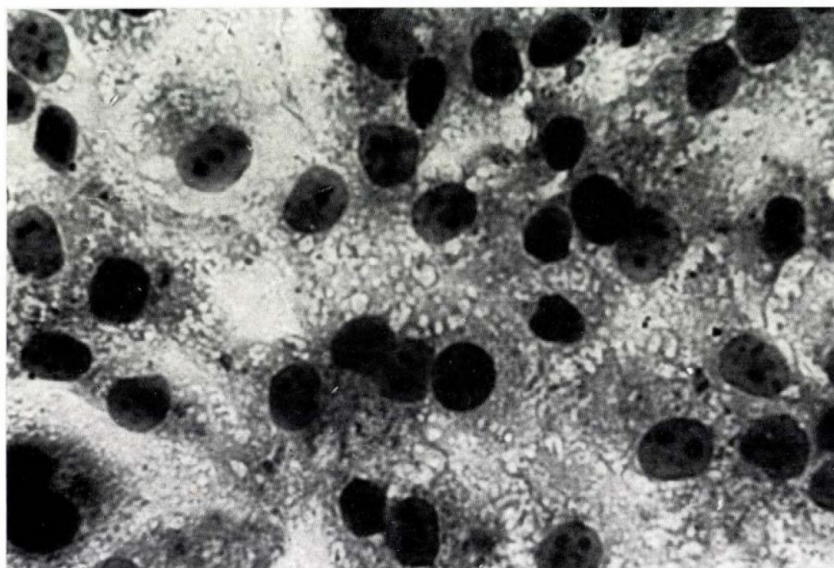


Fig. 4. Cells incubated in 10^{-7} M arachidic acid in HBS for 30 min. at 37°C and washed and reincubated in HBS + Dowex beads for an additional 30 minutes. Cell margins poorly distinguishable, cytoplasm clear and slightly foamy with small vacuoles. Practically all cells contain some large, confluent vacuoles along their edges (3+)

BA exhibited vacuoles in a characteristic localization. The general appearance of the monolayer was that of a control. At the margins, however, along the free edges of the cells numerous, large, spherical vacuoles with clear, homogeneous contents were observed (Fig. 8). Some of the cells incubated with Dowex granules in HBS for 90 minutes exhibited 10—15 randomly distributed small vacuoles varying in diameter and of definitely structured (packed beads) contents. The normal cytoplasmic granulation was preserved. These cells had no special localization within the monolayer (Fig. 9.)

In the next experiment sets of cultures were pretreated each for 30 minutes at 37°C with HBS containing $3 \times 10^{-5}\text{ BA}$ or 10^{-9} to 10^{-5} M arachidic or palmitic acid. Incubation was followed by rinsing with excess HBS, and then Dowex granules in HBS were added. Part of the identically treated cultures was incubated for 30, part for 60 minutes.

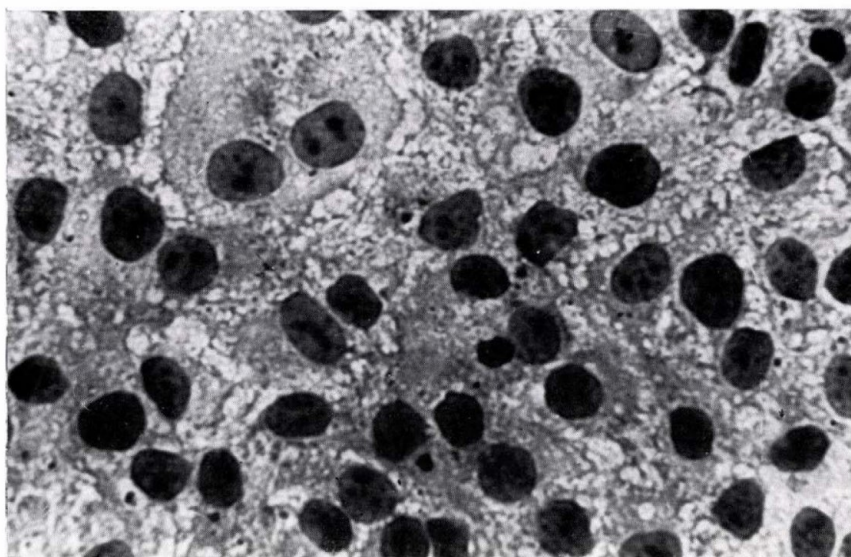


Fig. 5. Cells incubated in HBS + Dowex beads for 90 minutes at 37° C. From the 60th minute on 10^{-7} M palmitic acid was also present. Cell margins hardly distinguishable, cytoplasm foamy, loaded with vacuoles varying in diameter. Numerous large, confluent vacuoles of various localization in the cytoplasm of all cells in the monolayer (4 +)

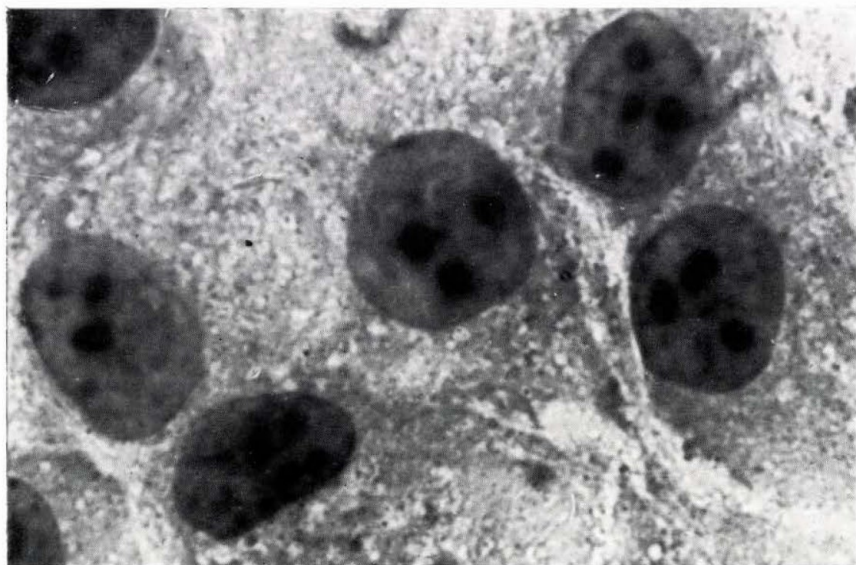


Fig. 6. Cells incubated for 90 minutes at 37° C in HBS. Cells in close contact within the monolayer. Cytoplasm granular, with occasional minute vacuoles

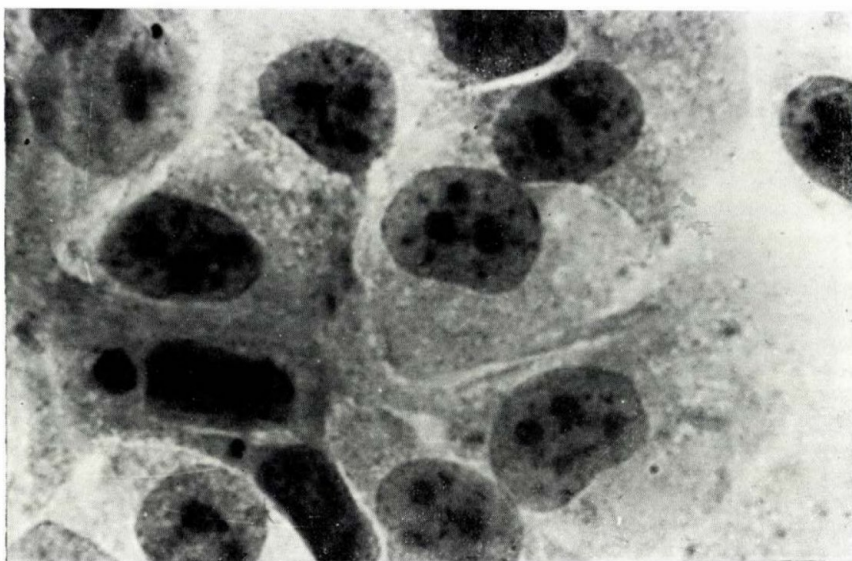


Fig. 7. Cells incubated for 90 minutes at 37° C in HBS + 10^{-5} M arachidic acid. Close packed cells within the monolayer. Cytoplasm clear, finely granular. Margins are poorly distinguishable



Fig. 8. Cells incubated for 90 minutes at 37° C in HBS + 3×10^{-5} M BA. Cells at the monolayer's edges. At the free margin of cells the cytoplasm is loose and exhibits several large, clear vacuoles. The cell's margins are clearly visible even in the region of extensive vacuolization

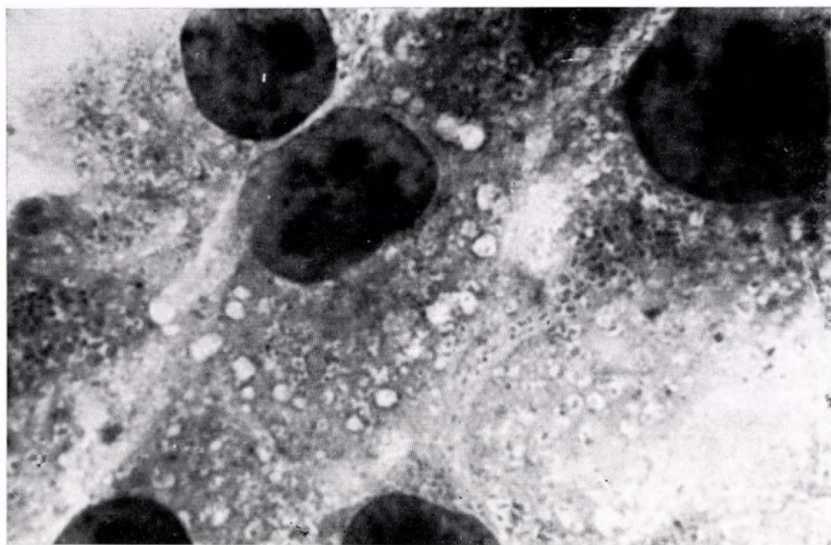


Fig. 9. Cells incubated in HBS + Dowex beads for 90 minutes at 37° C. Close packed cells within the monolayer. Margins poorly defined, cytoplasm granular. Peripherally, vacuoles of medium size, with irregular margins and structured contents. Minute, clear vacuoles are scattered randomly in the cytoplasm.

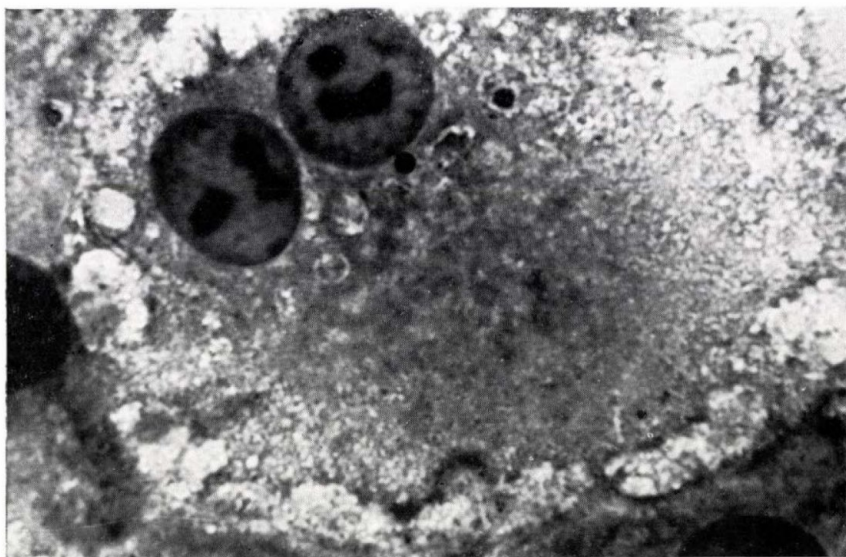


Fig. 10. Cells incubated in HBS + Dowex beads for 90 minutes at 37° C. At the 60th minute of incubation 10^{-5} M palmitic acid was added to the system. A cell with two nuclei is shown. The cytoplasm is loaded with small vacuoles throughout. From the large, confluent peripheral vacuoles filled with granules the latter seem to stream towards the nuclei.

Table I
Stimulation of phagocytosis by bovine albumin and fatty acids

Hanks' solution supplemented with	Grade of vacuolization (90 minutes at 37° C)			
	Control	Preincubation with supplement 30 min.		Dowex present 0 to 90 min. Supplement added at 60 min
		Dowex present from the		
		30th to 60th min.	30th to 90th min.	
0	0	1+	2+	2+
Bovine albumin $3 \times 10^{-5} M$	2+ (exclusively marginal)	2+	3+	4+
Arachidic $10^{-5} M$	0	3+	4+	4+
$10^{-7} M$	0	3+	4+	4+
$10^{-9} M$	0	4+	4+	4+
Palmitic acid $10^{-5} M$	0	4+	4+	4+
$10^{-7} M$	0	4+	4+	4+
$10^{-9} M$	0	4+	4+	4+

After 60 minutes practically all cells in all cultures pretreated with any of the stimulants had engulfed masses of granules. Their cytoplasm appeared merely as a network surrounding the bead-loaded vacuoles. Phagocytosis in 30 minutes was somewhat less intensive by cells pretreated with arachidic acid at high concentration and relatively poor in those pretreated with BA.

In another experiment stimulants were added in the 60th minute of incubation with Dowex beads in HBS. The time of the simultaneous presence of beads and stimulants was 30 minutes. The aim was to detect the possible inhibitory effect of adsorbed beads on the stimulants' action.

No inhibition was observed and the cells became filled with granules in 30 minutes. The effect of all the stimulants studied was identical (Fig. 10).

A survey of the results obtained is given in Table I.

Discussion

Various substances have been shown to induce or stimulate phagocytosis or pinocytosis in all sorts of protozoan and metazoan cells [1, 3, 7, 8]. The active substances were anionic, the inactive or poorly active ones cationic and neutral [1]. We studied two sorts of anionic molecules *viz.* BA and fatty acids.

BA is a large molecule with several possible conformations and the capability to adsorb or bind firmly a variety of molecules and ions, including fatty acids. The rapid adsorption and desorption of BA to cells *in vitro* has been demonstrated [9]. Cohn [1] has shown the pinocytosis stimulating effect of BA on cultured freely moving mouse macrophages. The mechanism of this effect is poorly understood.

In our experiments monolayers of monkey kidney epithelial cells were used. In such cultures outgrowth occurs at the periphery, while cells in the monolayer are non-motile ("contact inhibition") [10]. The membrane of a cell along its contact is supposed to consist mainly of a bimolecular lipid layer, while free edges with processes and pseudopodia have membranes of globular micellar structure [11]. The latter involves enhanced activity *i.e.* growth, movements, pinocytosis, phagocytosis, etc.

These facts appear to explain the mechanism of formation of peripheral vacuoles in cells of PMK III/1 monolayers treated with BA (Fig. 8).

The other type of anionic molecules used were fatty acids. Both palmitic and arachidic acid have a $-\text{COO}^-$ group as the hydrophilic part of the molecule and a C_{16} and C_{20} hydrocarbon chain as their respective hydrophobic parts. Such molecules have been found by HAYDON and TAYLOR [12] to be accommodated in an oriented way by bimolecular lipid leaflets. They were also shown to be metabolized at a considerable rate [3]. The vague contours and the light cytoplasm of fatty acid treated cells are supposed to be signs of these phenomena (Fig. 7).

Both types of substance equally stimulated uptake of Dowex beads. Thus their effect on phagocytosis as well as on pinocytosis [1] appeared to involve a mechanism of moderate specificity. With fatty acids in the range tested neither the concentration, nor the chain length, nor the time of addition appeared to influence their phagocytosis stimulating effect.

Other studies from this laboratory [14] yielded evidence of increased efficiency of infection of cells with poliovirus following the addition of fatty acids. The effect depended strictly on concentration, chain length and time of addition of fatty acids. Thus it appeared that the mechanisms of the infection enhancing and the phagocytosis stimulating effects of fatty acids were different.

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STREPTOMYCIN-LIKE COMPOUND ENZYMICALLY RELEASED FROM A STREPTOMYCIN-NON-PRODUC- ING STREPTOMYCES GRISEUS STRAIN

By

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(Received September 1, 1967)

Summary. A substance of antibiotic activity was released by lysozyme from the mycelia of a streptomycin-non-producing *Streptomyces griseus* strain. The digestion product was identified by paper chromatography. *B. megatherium* KM strain dependent on streptomycin and *B. subtilis* strains resistant and sensitive to streptomycin were also used for identification. These investigations showed that a streptomycin-like material was released by lysozyme digestion.

Picric acid hydrolysis of the mycelia of the streptomycin-non-producing *Streptomyces griseus* also resulted in a substance of antibiotic activity.

Correlation between streptomycin producing ability and the carbohydrate content of the isolated cell walls of *Streptomyces griseus* strains has also been investigated and is discussed.

The cell wall composition of streptomycin producing and streptomycin-non-producing *Streptomyces griseus* strains was compared in previous experiments [1, 2]. Streptidine, a component of the streptomycin (SM) molecule, was detected and identified in the isolated cell wall of streptomycin-non-producing *Streptomyces griseus* strain No.45 [3]. The problem arose whether the streptidine had originated from the SM molecule, the latter being decomposed under the conditions of the isolation. The cell wall of the non-producing strain had namely been treated with 12% HCl for three hours at 100°C, to liberate the streptidine from the wall structure. During the hydrolysis the SM — had it been present in the wall structure — would have been decomposed to its constituents streptidine, streptose, N-methyl-glucosamine.

To elucidate the problem, milder hydrolysis circumstances were employed, under which streptomycin does not decompose.

Digestion with lysozyme proved convenient for hydrolysis as the cell wall of strain No. 45 was sensitive to it.

Materials and methods

Strains, media and cultivation. The characteristics and cultural behaviour of the *Streptomyces* (*Str.*) *griseus* strains, and the composition of the soya-bean medium used in the experiments were described previously [4].

Isolation and purification of the cell walls were carried out by the previously applied method [2].

Hydrolysis, and the determination of hexose and pentose were accomplished as described previously [2].

Hexosamine content of the isolated cell wall was determined by the method of Boas [5].

Paper chromatography. Whatman's No. 1 filter paper and the following solvent systems were employed [6]: n-butanol saturated with water; 40 ml n-butanol, 10 ml methanol, 20 ml water; 100 ml of 80% aqueous methanol, 10.5 ml piperidine (adjusted to pH 9.0–9.5 with acetic acid); 50% acetone solution; n-butanol saturated with water containing 2% (w/v) p-toluene-sulphonic acid.

Picric acid hydrolysis. The hydrolysis procedure was a slight modification of the method introduced by HOLDSWORTH [7].

Forty-three g of mycelia (wet weight) of the SM-non-producing strain No. 45 was combined with 100 ml of concentrated picric acid. After 10 minutes heating at 100°C it was filtered immediately through glass filter (G5). The filtrate was put aside and the remainder of mycelia was treated further with 100 ml of concentrated picric acid at 100°C for 10 minutes and filtered again immediately. This procedure was repeated 5 times. The filtrates were evaporated separately to 10 ml and to each of them 100 ml of acetone was added. Yellowish crystals precipitated instantly. They were filtered out, taken up in 0.067 M phosphate buffer (pH 7.8) and tested for antibiotic activity against *B. subtilis* strains. 0.5 ml of concentrated picric acid solution in 0.5 ml of 0.067 M phosphate buffer (pH 7.8) served as control, but it was not inhibitory against *B. subtilis*.

Lysozyme treatment. The mycelia were washed three times with distilled water. The washed mycelia were suspended in 0.067 M phosphate buffer pH 6.6 containing lysozyme (Fluka 5×recryst.) at a final concentration of 100 µg/ml.

Disintegration of mycelia. The mycelia were washed three times with distilled water and disrupted mechanically in an apparatus prepared in our laboratory [8]. Disintegration was performed at 6°C for 45 minutes, to obtain effective disruption.

Results and discussion

Strain No. 45 was cultivated in soya-bean medium in submerged culture. Mycelia of different ages were harvested and treated with lysozyme as described in Materials and methods.

Table I shows the antibiotic activity of products liberated by lysozyme from the streptomycin-non-producing *Str. griseus* strain No. 45 in 12 to 13 hours. Digestion with lysozyme for 1 hour did not liberate substances of antibiotic activity. Lysozyme alone under the same conditions had no antibiotic effect, nor had the mycelia when treated without lysozyme under the same conditions. Thus, the release of antibiotic substances was the result of lysozyme digestion.

In the following, 72 hours old mycelium digested for 13 hours with lysozyme (72/13) was studied. The antibiotic was identified by paper chromatography and by applying a streptomycin dependent bacterium.

The antibiotic of fraction 72/13, investigated by paper chromatography, behaved similarly to SM-sulphate, giving the same R_f values in the five solvent systems described under Materials and methods.

Bacillus megatherium KM, dependent on SM, grew on medium containing fraction 72/13 like in the presence of 0.1–1.0 µg/ml SM.

To decide whether the SM molecule was released from the cell wall structure or had been present in the pool of the mycelium of strain No. 45 mycelia of the same dry weight as those subjected to enzymic digestion, were disrupted mechanically at 6°C and centrifuged at 2400 g for 20 minutes.

Table I

Effect of lysozyme on mycelia of SM-non-producing strain No. 45

Age of mycelia (hours)	Treatment with lysozyme at 37°C (hours)	Antibiotic activity against <i>B. subtilis</i> , sensitive to SM	Antibiotic activity against <i>B. subtilis</i> resistant to SM
24	0*	—	—
24	0**	—	—
24	2	—	—
24	12	±	—
48	0*	—	—
48	0**	—	—
48	1	—	—
48	12	+	—
72	0*	—	—
72	0**	—	—
72	13	++	—
72	13***	—	—
Streptomycin sulphate (10 µg/ml)		++	—

* Samples taken before adding lysozyme.

** Samples taken immediately after adding lysozyme.

*** Experiments made under the same conditions in the absence of lysozyme.

+ Inhibition zone between 11–12 mm (hole diameter 10 mm).

++ Inhibition zone between 17–19 mm (hole diameter 10 mm).

Each sample was centrifuged at 2400 g for 20 minutes, the freeze dried supernatants were resuspended in 2 ml of 0.067 M phosphate buffer (pH 7.8), and tested against *B. subtilis*.

The supernatants were freeze dried, treated with lysozyme and tested against *B. subtilis*. Traces of antibiotic activity were only observed. These experiments indicated that the SM-like substance was released by enzymic hydrolysis of the mycelial wall of SM-non-producing *Str. griseus* strain No. 45, and originated mostly from the lysozyme digestion process.

We have succeeded in liberating substances of antibiotic activity by picric acid hydrolysis of the mycelia of SM-non-producing strain No. 45. It is known that streptomycin, being of basic character, readily forms picrate salts of antibiotic effect.

The SM-producing *Str. griseus* strain No. 52-1 was not treated with lysozyme to release SM from its structure, as the appearance of SM would not have supplied evidence of the structural origin of the antibiotic.

The SM molecule contains hexosamine in the form of N-methyl-glucosamine. Lysozyme is known to attack the bonds between the acetyl-amino-sugars and to liberate hexosamine-peptides from the cell wall [9]. On the

Table II/a

Carbohydrate content of the isolated cell wall of Str. griseus No. 52-1

Hexose content (determined by the anthrone reaction), mg/100 mg dry weight of cell wall	Pentose content (determined by the orcinol test), mg/100 mg dry weight of cell wall	Hexosamine content (determined by the method of Boas), mg/100 mg dry weight of cell wall	Age of strains (hours)
10.87	1.17	—	16
—	—	3.15	20
17.02; 18.90; 23.40; 15.90	2.63; 1.26; 2.02; 1.29	—	48
—	—	10.0	96

Table II/b

Carbohydrate content of the isolated cell wall of Str. griseus No. 45

Hexose content (determined by the anthrone reaction) mg/100 mg dry weight of cell wall	Pentose content (determined by the orcinol test) mg/100 mg dry weight of cell wall	Hexosamine content (determined by the method of Boas), mg/100 mg dry weight of cell wall	Age of strains (hours)
12.35	3.50	—	10
11.51	2.20	—	14
—	—	2.75	24
13.45; 9.34	1.79; 2.34	—	48
—	—	1.65	69

basis of these observations and of our previous experiments [1, 2, 3, 10] the eventual correlation between the carbohydrate content of the cell walls of *Str. griseus* strains and their SM-producing ability was investigated.

Isolated and purified cell walls [2] of a SM-producing *Str. griseus* strain No. 52-1 and of its SM-non-producing mutant No. 45 were used for these investigations.

As Table II/a shows, the hexose and pentose content of the cell wall of strain No. 52-1 increases with the age of the mycelia. This increase is especially high in the case of hexosamine. The hexosamine content of the cell wall of a 96-hour-old strain is almost three times higher than that of cell walls of 20-hour-old mycelia. The changes in pentose-content are insignificant.

According to the data of Table II/b, the hexose, pentose and hexosamine content of strain No. 45 did not change significantly. Its pentose concentration was, however, high, and the hexose : pentose ratio approximately 2.5 times higher than in the cell wall of SM-producing strain No. 52-1 [2].

The presence of streptidine, a component of the SM molecule, was detected in the cell wall of the SM-non-producing strain [3] and later in the cell

wall of SM-producing strain [10]. We could not estimate the streptidine content of the isolated cell wall quantitatively in view of the small amount at our disposal and of the low sensitivity of the quantitative SAKAGUCHI colour-reactions that served to detect streptidine [11, 12, 13].

We maintain our theory [3, 10] that streptidine has some role in the cell wall synthesis of *Str. griseus* strains, but the proposed correlation of its presence in the cell wall and the inability to excrete SM are to be questioned. On the basis of the present experiments we suppose that even the whole SM molecule, or a SM-like molecule, may be present in the cell wall structure. There are differences between the cell wall composition of SM-producing and non-producing *Str. griseus* strains. The non-producing strain has a relatively high pentose content, and the producing strain increases its hexosamine content significantly with age. Rhamnose, the pentose sugar present in the cell wall of our strains [2], could be converted into streptose by *Str. griseus* [14], and glucosamine, the other sugar component of the SM-molecule, is also present in the cell wall [2]. It is not known what correlation exists between the different carbohydrate compositions of the cell walls and the SM-producing ability of the SM-producing and non-producing strains.

The relationship between cell wall structure and ability to produce SM becomes more complicated if we take into account the morphological difference between the cell walls of the SM-producing strain No. 52-1 and those of the SM-non-producing strain No. 45. Microscopic and electron microscopic observations showed that the non-producing strain forms cross-walls, the frequency of which increases with age. The SM-producing strain does not or very rarely forms cross-walls. Thin sections for electron microscopy proved also that the non-producing strain has thicker cell walls than the SM-producing strain. The thickness of the cell wall increases with age. These observations have been supported by the data of Table III.

Table III

Yield of cell wall prepared from mycelia of different ages

Strains	Age of strains (hours)	Wet weight of mycelia (g)	Dry weight of isolated, purified and freeze dried cell wall (g)	Yield of cell wall (%) [*]	Rate of increase of cell wall yield
45	24	385	0.161	0.0418	16.54
45	69	48	0.332	0.6916	
52-1	20	83	0.140	0.1686	2.40
52-1	96	62	0.251	0.4048	

^{*} The percentage of yield of cell wall is of comparative value. For calculations the wet weight of mycelia and the dry weight of cell walls were taken into consideration.

Applying the same isolation and purification method for obtaining the cell walls, there was a marked difference in the yield. The rate of increase of the cell wall depending on age was 16.5 times higher in *Str. griseus* strain No. 45, while only 2.5 times higher with the SM-producing strain No. 52-1. On the basis of these data and of our previous observation that streptidine was a constituent of the cell wall of *Str. griseus* strains [3, 10], we suppose that the SM-like substance or SM itself is a constituent of the normal cell wall structure.

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INDUCTION AND MULTIPLICATION OF λ -PHAGE

II. THE EFFECT OF VALINE AND LEUCINE

By

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Summary. Phage production after mitomycin induction takes place in the shift down state before the start of multiplication in the uninduced culture. Valine and leucine inhibit phage production at concentrations inhibiting cell multiplication. If the inhibitory effect of the above amino acids is suspended by the addition of casein hydrolysate, the lag-phase of phage production considerably shortens as compared with the induction made simultaneously with addition of casein hydrolysate. This may reflect that several steps of phage-multiplication take place during the inhibition.

In a previous paper we have reported [1] that in the shift down state on the addition of mitomycin the prophage dissociates from the chromosome of the host cell without protein synthesis and in the presence of chloramphenicol (CM) the dissociated prophage maintains its primary activity for a long time. The growth of the *Escherichia coli* K 12(λ 28) strain used in our experiments is inhibited by valine [2] and in the shift down state also by leucine [3]. Thus, it seemed interesting to study whether in the presence of these amino acids mitomycin induction could take place, and how far the dissociated prophage would develop.

Materials and methods

In the experiments essentially the same methods were used as previously [1].

Strains. *E. coli* K 12(λ 28) was used as lysogenic strain. Phage-determinations were carried out with *E. coli* C 600 strain.

Nutrient media. As minimal medium that of DAVIS and MINGIOLI [4] complemented with 1 ml 0.01% casein hydrolysate per litre (Bacto casamino acid "Difco") was used. This extremely small amount of casein hydrolysate was necessary for the following reason. When the washed bacterium suspension was inoculated into a minimal medium not containing this minimal casein hydrolysate, the lag-phase of phage-production following induction varied considerably; it was sometimes prolonged to 200 minutes and sometimes could not be detected at all.

As complete medium, the above minimal medium completed with 0.25% casein hydrolysate was used. In certain cases, when completion of the minimal medium was not performed simultaneously with the inoculation, the amount of casein hydrolysate added was identical to the above concentration.

Experimental conditions. Preparation of culture in the shift down state. A 16-hour-old bouillon culture was diluted 1 : 10 with the same medium and after incubating for two hours the cells were washed twice and suspended in minimal medium at a concentration of 10^6 cells/ml. The cultures were incubated at 37°C. For induction, 1 μ g/ml of mitomycin C was added at zero or 90 minutes.

Phage determination was carried out at 37°C. Cultures were treated with chloroform at least for two minutes at 2°C, as described previously.

Results

Phage production started after 90 minutes (Fig. 1) following induction in the shift down state considerably earlier than did the increase of the viable cell count, in the control culture, because the lag-phase of the shift down takes about three hours under our experimental conditions. If the shift down state was suspended by the addition of casein hydrolysate or the induced

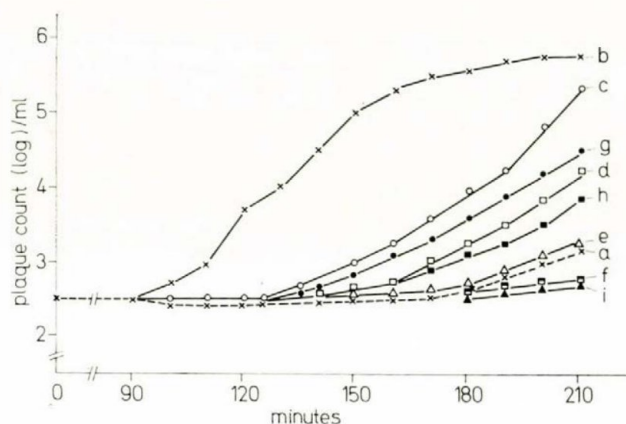


Fig. 1. Inhibitory effect of leucine and valine on induced phage production in the shift down state in minimal medium. Cultures induced with 1 µg/ml mitomycin C. Inoculum grown on bouillon. Symbols: a = not induced culture on minimal medium; b = induced culture on minimal medium; c = induced culture on minimal medium containing 0.5 µg/ml L-leucine; d = induced culture on minimal medium containing 1.0 µg/ml L-leucine; e = induced culture on minimal medium containing 2.5 µg/ml L-leucine; f = induced culture on minimal medium containing 25 µg/ml L-leucine; g = induced culture on minimal medium containing 0.05 µg/ml L-valine; h = induced culture on minimal medium containing 0.1 µg/ml L-valine; i = induced culture on minimal medium containing 1.0 µg/ml L-valine

culture was grown on minimal medium, phage production started between 40 to 50 minutes, after this period the phage count of the induced culture exceeded that of the not induced cultures.

The effect of L-amino acids on phage production induced by mitomycin up to 200 µg/ml concentration was studied in the shift down state. Valine, leucine, serine, threonine, cysteine and methionine inhibited phage production (Table I). In earlier experiments [3] we studied the inhibitory effect of L-amino acids on the growth of *E. coli* K 12(λ 28) and found that the same amino acids inhibited growth and phage production, only cysteine acted differently; it inhibited phage production completely but failed to affect cell multiplication.

The growth of *E. coli* K 12(λ 28) strain was inhibited of valine at low concentrations [2] and in the shift down state also by leucine. As seen from Table I, these small amounts of valine and leucine inhibited phage multiplication and cell multiplication very similarly.

Similarly to growth valine inhibited phage production, too, when the inoculum was grown on minimal medium. In the same inoculum the inhibitory effect of leucine on growth and phage production was one order of magnitude less (Figs 1, 2).

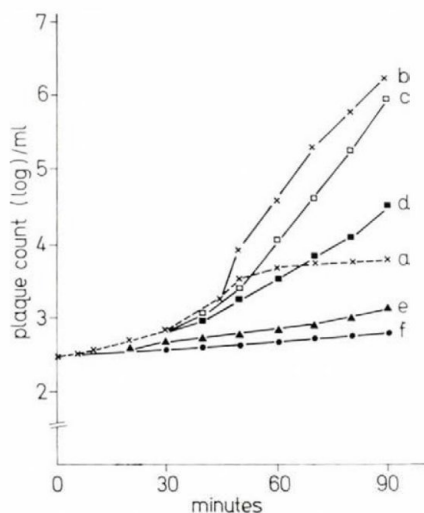


Fig. 2. Inhibitory effect of leucine and valine on induced phage production without shift down state in minimal medium. Cultures induced with 1 $\mu\text{g/ml}$ mitomycin C. Inoculum grown on minimal medium. Symbols: a = not induced culture on minimal medium; b = induced culture on minimal medium; c = induced culture on minimal medium containing 25 $\mu\text{g/ml}$ L-leucine; d = induced culture on minimal medium containing 0.1 $\mu\text{g/ml}$ L-valine; e = induced culture on minimal medium containing 1.0 $\mu\text{g/ml}$ L-valine; f = induced culture on minimal medium containing 5.0 $\mu\text{g/ml}$ L-valine

Table I

Inhibitory effect of amino acids on induced phage production

	Relative phage concentration		
	2.5 $\mu\text{g/ml}$	25 $\mu\text{g/ml}$	200 $\mu\text{g/ml}$
	amino acid		
L-leucine	0.1	0.01	0.01
L-valine	0.1	0.01	0.01
L-isoleucine	10.0	1.25	0.2
L-methionine	100.0	40.00	3.5
L-cysteine HCl	40.0	1.00	0.01
L-serine	1.5	0.04	0.01
L-threonine	0.03	0.01	0.01

After 180 minutes incubation the plaque count was determined after treatment with chloroform. Values represent the percentage of the phage concentration of amino acid free control.

Next, it was attempted to obtain information concerning the character of the inhibitory effect on phage multiplication of valine and leucine. If the shift down state was blocked by the addition of casein hydrolysate in the 90th

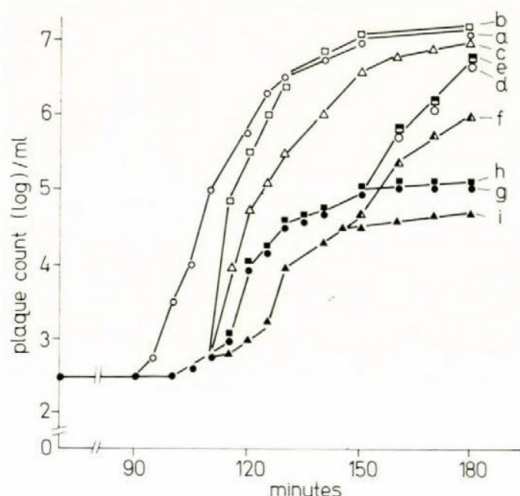


Fig. 3. Lag phase of induced phage production after suspension of amino acid inhibition. Experiment started in the shift down state. Suspension of the shift down state and of the inhibitory effect of leucine and valine was accomplished by the addition of 0.25% casein hydrolysate at the indicated time. Cultures induced with $1 \mu\text{g/ml}$ mitomycin C. Inoculum grown on bouillon. Symbols: a = culture induced at 0 time on minimal medium, addition of casein hydrolysate at 90 minutes; b = at 0 time induced culture on minimal medium containing $1 \mu\text{g/ml}$ L-valine, addition of casein hydrolysate at 90 minutes; c = culture induced at 0 time on minimal medium containing $2.5 \mu\text{g/ml}$ L-leucine, addition of casein hydrolysate at 90 minutes; d = culture preincubated on minimal medium induced at 90 minutes, simultaneous addition of casein hydrolysate at 90 minutes; e = culture preincubated on minimal medium induced at 90 minutes, simultaneous addition of casein hydrolysate; f = culture preincubated on minimal medium containing $1.0 \mu\text{g/ml}$ L-valine, induced at 90 minutes, simultaneous addition of casein hydrolysate; g = culture preincubated on minimal medium containing $2.5 \mu\text{g/ml}$ L-leucine, induced at 90 minutes, simultaneous addition of casein hydrolysate; h = culture preincubated on minimal medium, addition of casein hydrolysate at 90 minutes; i = culture preincubated on minimal medium containing $2.5 \mu\text{g/ml}$ L-leucine, addition of casein hydrolysate at 90 minutes

minute and the culture was simultaneously induced by mitomycin, the induced phage production started after 60 minutes. Exactly the same result was obtained when $1 \mu\text{g/ml}$ valine or $2.5 \mu\text{g/ml}$ leucine was added to the culture. If induction was performed at zero minute in the presence of these amino acids, and their effect and the shift down were suspended in the 90th minute with casein hydrolysate, the induced phage production started in 10–15 minutes (Fig. 3). When induction with mitomycin was performed in the presence of CM at zero minute and the shift state was suppressed in the 90th minute by casein hydrolysate, and to eliminate the effect of CM the culture

was diluted 200-fold with preheated medium, induced phage production started after 20 to 25 minutes, thus later than in the cultures induced in the presence of leucine or valine (Fig. 4). After the elimination of CM the non-induced

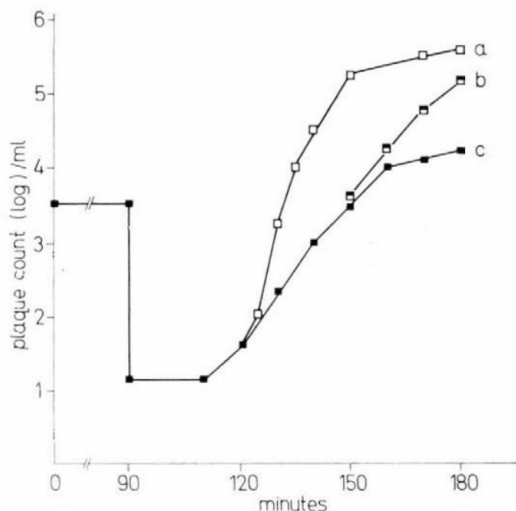


Fig. 4. Lag phase of induced phage production after suspension of CM inhibition. Experiment started in the shift down state on minimal medium containing 20 $\mu\text{g/ml}$ CM, inoculated with 10^7 cells/ml grown on bouillon. Suspension of shift down state and CM inhibition at 90 minutes, by 1 : 200 dilution and simultaneous addition of 0.25% casein hydrolysate. Cultures induced with 1 $\mu\text{g/ml}$ mitomycin C.

Symbols: a = culture induced at 0 time; b = culture induced at 90 minutes; c = not induced culture

culture exhibited a higher phage count on complete medium than without CM treatment under similar conditions.

Discussion

Numerous papers have dealt with the effect of the shift down on phage production. According to BEN GURION [5], the multiplication of phage is inhibited in the shift down state only in the case of infection but not in that of induction. In contrast, GOTS and HUNT [6] observed the inhibition of phage production in the shift down state by inducing it with UV irradiation.

FOWLER and COHEN [7] reported on the action of certain amino acids upon T2 phage multiplication in the shift down state. They found that leucine and serine had an inhibitory effect. In our experiments several amino acids were found to inhibit the multiplication of mitomycin-induced λ phage in the shift down state. Among these amino acids, valine, leucine, serine and threonine had an intensive effect, only in the case of cysteine could no paral-

lelism be detected between its effect on bacterial growth and induced phage production.

Our experiments have elucidated the nature of the phage multiplication inhibitory effect. After induction with mitomycin prophage dissociation takes place immediately in a part of the culture also in the shift down state [1]. The complete phages appear after 90 minutes (see Fig. 1), the period necessary for a complete vegetative cycle under these conditions. If induction is performed in the presence of valine and leucine and the inhibitory effect together with the shift down state is suspended in the 90th minute, induced phage production soon starts. In contrast, if induction is performed in the 90th minute, when the inhibition by valine and leucine is suspended, induced phage production starts only after a longer period. This refers to the possibility that several steps of phage production take place during the period of inhibition. It seems probable that the process requiring time after prophage dissociation is derepression, as indicated by the experiments with CM, and at the same time it follows that the derepression after mitomycin induction, in contrast to induction with UV irradiation [8] does not require protein synthesis. Furthermore it seems that in the presence of CM the synthesis of early messenger RNA takes place. After the inhibition by valine and leucine has been suspended, the starting period of phage production is shorter than after the transitory effect of CM. This difference might mean that the inhibition of phage synthesis by amino acids, as compared to the effect of CM, points to a later phase of the phage replication cycle; early protein synthesis and probably the late messenger RNA synthesis, too, take place.

The mechanism of the growth inhibitory effect of valine is well known [9, 10]. Valine inhibits the activity of the first common enzyme of valine-isoleucine biosynthesis, causing thus an isoleucine deficiency. This suggests that the enzyme necessary for the replication of phage DNA is formed from the isoleucine pool even in the presence of valine. However, secondary protein synthesis which requires a considerable amount of isoleucine, does not take place.

The growth inhibitory effect of leucine is not clear. It might have some connection with the interaction of protein and RNA synthesis [11, 12]. In the above experiments leucine behaved similarly to valine in respect of phage production, thus the effect of leucine manifests itself with the inhibition of protein synthesis.

It should be emphasized that, similarly as in our experiments with CM [1], in the case of valine and leucine, the prophage dissociated on the addition of mitomycin and maintained its primary activity for several hours.

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FIXATION AND SENSITIZING CAPACITY IN THE GUINEA PIG OF HORSE IgA, RABBIT AND CHICKEN IgG

By

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Summary. It has been shown that rabbit IgG, horse IgA and chicken IgG are fixed in the tissues of the guinea pig. Rabbit IgG was demonstrated in the lung in larger amounts than in other organs. The antibody content increased in all organs when horse IgA or chicken IgG were given in larger doses. Although increased doses of these antibodies produced the same level in the lung as rabbit IgG, they did not sensitize the animals. Fatal shock developed only after a challenge with rabbit IgG. The findings indicate that the sensitizing capacity of antibodies is not associated with the degree of fixation in the tissues.

It has long been recognized that the guinea pig can be sensitized passively with rabbit, dog, monkey and human immune serum but not with horse, goat, pig, rat or fowl immune serum. Studying the cause of this phenomenon, HUMPHREY and MOTA [1] have shown that rabbit, horse and rat gamma globulins are fixed *in vitro* to the mesentery of guinea pigs. SÓNAK [2] demonstrated in passive and inverse anaphylaxis that normal and immune rabbit globulins given intravenously were both fixed in various organs of the guinea pig, but experiments with chicken and pig globulins failed to show any fixation. It was concluded that fixation of globulins in guinea pig tissues depends on species-specific rather than on immunogenic properties. Accordingly, if the guinea pig's tissues fail to bind the normal globulins of a given species, they will not fix immune globulins of the same species and thus they will not be sensitized. That means that there is a close association between the sensitizing power and fixation capacity of heterologous antibodies. KESZTYÜS *et al.* [3] showed that the lung of the guinea pig sensitized passively with rabbit antiovalbumin immune serum fixes 3 to 4 times as much homologous antigen as the same organ of the control animal. Since in the antigen content of other organs there was no difference between sensitized and control animals, they concluded that the shock organ of the guinea pig fixes the sensitizing heterologous antibody in considerably larger amount than other organs.

In the experiments described in this paper antibodies from rabbit, horse and chicken immune sera were isolated, labelled with radioactive isotope and used for the sensitization of guinea pigs. The method allowed a direct measurement of the localization of antibodies *in vivo* and an estimation of the connection between sensitizing capacity and the amount of fixed antibodies.

Materials and methods

Antigens. (a) Ovalbumin (OA) was recrystallized three times and gel-filtered in our Institute. (b) Human serum albumin (HSA) was obtained from the Institute for Serobacteriological Production and Research "Human", Budapest; gel-filtration of the preparation was performed in our Institute.

Antisera. (a) Serum of rabbits hyperimmunized with OA. (b) Serum of horses hyperimmunized with human serum (Institute for Serobacteriological Production and Research "Human", Budapest). (c) Serum of chickens hyperimmunized with HSA.

Labelling of antisera. The sera were labelled with ^{131}I as described by TALMAGE *et al.* [4] prior to isolation of the antibodies, as preliminary experiments indicated that the antibody was more considerably damaged if the procedure was carried out with isolated antibodies.

Isolation of antibodies. Antibodies from ^{131}I -labelled rabbit, horse and chicken immune sera were precipitated with OA and HSA. The amount of antigen needed for a complete removal of antibodies was determined previously with quantitative precipitation. The complexes were dissociated with *N* acetic acid. The antibodies were separated from the antigens on Sephadex G-200 gel [5].

Quantitative precipitation of rabbit and horse immune sera was performed by HEIDELBERGER's method, that of chicken sera in 11% NaCl at pH 7.4 [6].

Passive sensitization. The first group of guinea pigs was sensitized intracardially with doses of $480\text{ }\mu\text{g}$ N/kg body weight of labelled and isolated rabbit anti-ovalbumin. Shock was elicited with the intrajugular injection of 4 mg OA. Animals belonging to the second group were sensitized as described above with 480 and $1500\text{ }\mu\text{g}$ N/kg doses of labelled and isolated horse anti-HSA. The animals were challenged after 24 and 48 hours with the intrajugular injection of 4 mg HSA. The third group was given $1500\text{ }\mu\text{g}$ N/kg doses of labelled and isolated chicken anti-HSA. An intrajugular challenging dose of 4 mg HSA was administered after 24 hours.

Determination of fixed antibodies. Two to five minutes after the eliciting injection, immediately before their death, guinea pigs sensitized with rabbit antibody were bled. Animals sensitized with horse and chicken antibodies were sacrificed by bleeding 10 minutes after the challenging injection. The lungs, liver, heart and ileum were removed, then the blood was blotted up and the gut was cleaned. Finally, 0.5 to 1 g portions of the organs were weighed, placed in serological tubes and counted by means of a Frieske-Hoeftner apparatus operating with an FH 421/26 type scintillation detector supplied with NaI(Tl)-Bohrloch crystal. The activity measured was compared with the activity of a ^{131}I -labelled antibody of known N content, then the antibody-N present in each organ was calculated. The organs were not completely cleaned of blood, as washing had been shown to remove part of the antibodies fixed to the tissues [7]. In calculating the values, the blood antibody content of the organs was, therefore, taken into correction.

Results

Figure 1 shows the elution according to molecular weight of the three isolated and labelled antibodies. Sedimentation rate of the isolated rabbit antibody as determined previously by ultracentrifugation was $s_{20,v} = 6.4\text{ S}$. Close before the peak given by rabbit IgG (I) was the elution maximum of horse IgA (II). Chicken IgG (III) preceded both of the above antibodies. Graphs for OA and HSA antigens are also presented. The isolated antibodies were identified by immune electrophoresis.

Pure labelled rabbit IgG and horse IgA were used for the intracardial sensitization of guinea pigs ($480\text{ }\mu\text{g}$ antibody-N per kg body weight). The homologous antigen was injected intrajugularly after 48 hours. Data for antibody-N values in the blood and organs are presented in Fig. 2.

It is seen that in animals sensitized with rabbit IgG the largest amount of antibody was fixed in the lung; the heart, liver and intestine contained

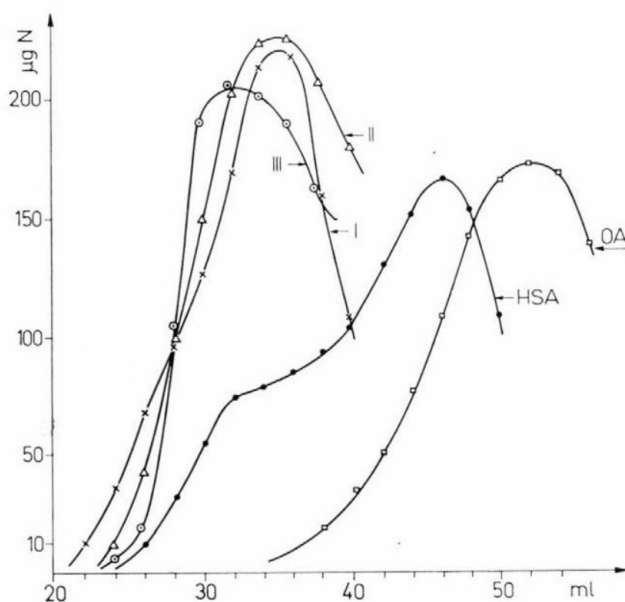


Fig. 1. Elution diagram of antibody-antigen complexes on Sephadex G-200 column.
I = rabbit anti-OA; II = horse anti-HSA; III = chicken anti-HSA

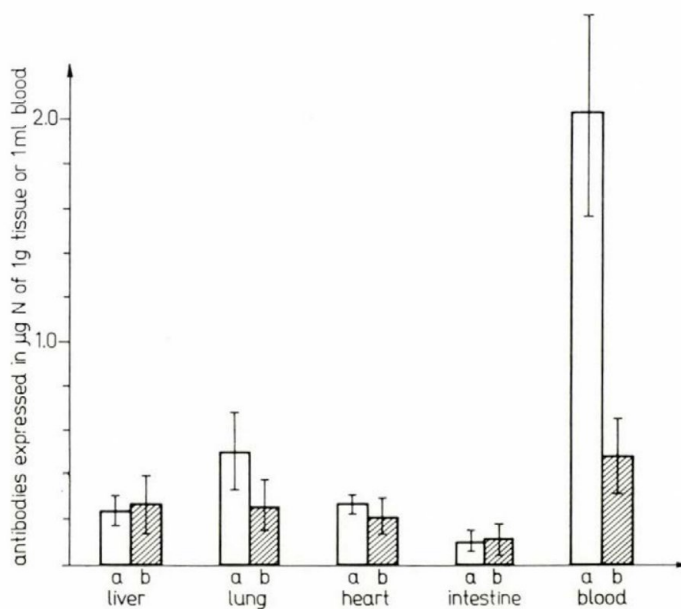


Fig. 2. Antibody-N content of guinea pig tissues 48 hours after the sensitizing injection.
a = 480 µg N/kg rabbit ¹³¹I-anti-OA; b = 480 µg N/kg horse ¹³¹I-anti-HSA

gradually lower amounts. The antibody content of blood was several times higher than that of the tissues. In guinea pigs sensitized with horse IgA we have found the most antibodies in the liver, the lung heart and intestine, in that order, contained gradually lower amounts. In 1 ml blood about twice as much antibody was shown as in 1 g liver. The difference in the fixation of the two kinds of antibodies was the most definite in the lung, where rabbit IgG content was significantly higher than the level of horse IgA. The liver, heart and intestine fixed both antibodies in about the

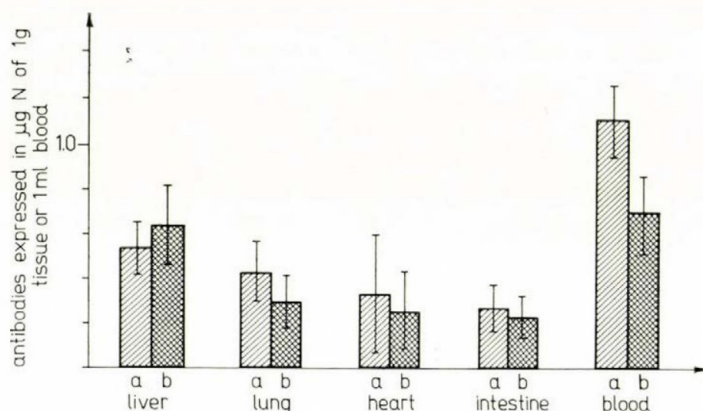


Fig. 3. Antibody-N content of guinea pig tissues 24 hours after the sensitizing injection. a = 1500 µg N/kg horse ¹³¹I-anti-HSA; b = 1500 µg N/kg chicken ¹³¹I-anti-HSA

same proportion. The blood level of horse IgA was considerably lower than that of rabbit IgG. The difference was explained by the fact that from the guinea pig horse immune globulin is excreted more rapidly than rabbit immune globulin [1].

In order to overcome difficulties due to the difference in biological half-life between horse IgA and rabbit IgG, another group of guinea pigs was sensitized with 3-fold doses (1500 µg N/kg) of horse IgA and estimations were performed 24 hours after the injection. As shown in Fig. 3, the level of circulating antibodies rose to twice the original but was still lower than the blood level in guinea pigs sensitized with 480 µg N/kg of rabbit IgG.

Figure 3 demonstrates further that the higher the circulating horse antibody level, the higher the antibody-N content of the tissues. Antibody fixation in the lung is, however, not increased as compared to that in liver, heart or intestine.

As the biological half-life in guinea pigs of fowl immune globulin is also shorter than that of rabbit immune globulins, a group of guinea pigs was immunized with 1500 µg N/kg chicken IgG. The homologous HSA antigen was injected after 24 hours then the animals were sacrificed. Fig. 3 shows

that chicken IgG was fixed in the tissues in proportions similar to horse IgA; the circulating amount of the former was significantly less than that of the latter.

The reaction to the challenging antigen of animals sensitized with various antibodies is presented in Table I. Fatal shock developed only in guinea pigs sensitized with rabbit IgG. Animals sensitized with 480 μg antibody-N/kg horse IgA showed no symptoms. Some guinea pigs which had been given 1500 μg antibody-N/kg horse IgA or chicken IgG, developed slight, uncertain symptoms after receiving the homologous HSA antigen.

Discussion

Immune-specific isolation, acetic acid treatment and gel-filtration did not affect the precipitating power of rabbit IgG [5]. Horse IgA and IgG treated in the same manner lost their precipitating capacity. At present we cannot explain this interesting phenomenon. KLINMAN *et al.* [8] were the first to show the difference between horse IgA and IgG: the former lost, the latter retained, the precipitating capacity after isolation. With immune-specific precipitation they isolated the two immune globulins and dissociated the antigen-antibody complex with excess hapten. They separated the two kinds of antibodies on DEAE cellulose. As their method did not include acetic acid treatment and gel-filtration, it is improbable that the precipitating capacity of our isolated horse IgA was lost as a result of these steps. KLINMAN *et al.* demonstrated that despite of a loss of its precipitating power, the isolated horse IgA retained its hapten-binding capacity.

TENENHOUSE and DEUTSCH [9] investigated the properties of chicken gamma globulin. They showed that physico-chemically this antibody stood nearer to mammals' IgA than to IgG. The precipitating power of the antigen was retained if chicken IgG was isolated from the immune serum on ion exchange resin. It has been described that considerably more antibody can be precipitated from chicken immune serum in 8 or 11 per cent NaCl than in physiological saline [6, 9].

In our experiments the association between sensitizing capacity and fixation of isolated antibodies has been examined. We have shown that rabbit IgG, a good sensitizing agent, reached a blood level of 1.5 to 2.5 μg N/ml 48 hours after the injection. Although it was demonstrated in all the examined organs, in the largest amount it was fixed in the lung, the shock organ of the guinea pig. Horse IgA, when given in the same sensitizing dose, reached after 48 hours a blood level as low as 0.3 to 0.6 μg N/ml. In the amount of fixed horse IgA there was no significant difference between the lung and other organs. In order to eliminate differences due to the different biological half-life of rabbit IgG, horse IgA and chicken IgG, horse and chicken anti-

Table I

Sensitizing antigens	Number of animals	Sensitizing dose, $\mu\text{g N/kg}$	Latency period (hours)	Eliciting dose	Symptoms
Rabbit anti-OA isolated on Sephadex G-200	1	480	48	4 mg OA	++++
	2				++++
	3				++++
	4				++++
	5				++++
	6				++++
	7				++++
	8				++++
	9				++++
	10				++++
Horse anti-HSA isolated on Sephadex G-200	11	480	48	4 mg HSA	—
	12				—
	13				—
	14				—
	15				—
	16				—
	18				—
	19				—
	20				—
	21				—
	22				—
	23	1500	24	4 mg HSA	+
	24				±
	25				+
	26				—
	27				++
	28				+
	29				+
	30				±
	31				+
	32				+
Horse anti-HSA	33	1500	24	4 mg HSA	—
	34				—
	35				—
	36				—
	37				—
Chicken anti-HSA isolated on Sephadex G-200	38	1500	24	4 mg HSA	+
	39				±
	40				+
	41				+
	42				±
	43				+
	44				+
	45				+
	46				—
	47				±

Symptoms: — = symptomless; ± = bristling of hair, scratching of nose; + = dyspnoea; ++ = staggering; ++++ = fatal shock.

bodies were given in sensitizing doses 3 times higher than rabbit IgG. It is known that immune globulin fixation in the tissues increases with the time; in our experiments horse IgA and chicken IgG reached the same tissue level as rabbit IgG did, but anaphylaxis occurred only in guinea pigs sensitized with rabbit IgG (see Table I). This finding indicates that the sensitizing power of antibodies does not depend on their fixation capacity. Our observations disagree, therefore, with the results of SÓNAK [2].

Increasing the sensitizing dose resulted in blood levels of 1 to 1.5 μg N/ml for horse IgA, and 0.8 to 1 μg N/ml for chicken IgG. The tissue level of these antibodies increased proportionally in all tissue samples. The antibody content of the lung was not higher than that of other organs.

Examining the function of rabbit IgG subunits, ISHIZAKA and ISHIZAKA [10] showed that the fragment carrying the antigenic property sensitized the skin of the guinea pig. Structures binding to tissues and fixing complement C' contained different disulphide bonds.

It may therefore be assumed that the different structure of subunits is responsible for the different behaviour of sensitizing and non-sensitizing antibodies.

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INCIDENCE OF RUBELLA VIRUS NEUTRALIZING ANTIBODY IN DIFFERENT AGE GROUPS

By

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Summary. A rubella neutralizing antibody survey has been performed. A total of 1937 serum samples was examined, covering the age groups from 0 to 80 years. Neutralization tests were carried out on RK-13 permanent rabbit kidney cell cultures by measuring inhibition of cytopathic effect.

The test sera were divided into two groups. Group I consisted of 1388 serum samples taken randomly from specimens submitted for routine laboratory examination. The donors were ranged into 11 age groups. The incidence of neutralizing antibodies was found to increase parallel with age. The first steep rise occurred in the 16—20-year age group, amounting to 74%. At 21—30 years, 85.5% of the population had antibodies. This level remained practically unchanged (81—88%) from 31 to 70 years of age.

In group II, a total of 549 blood samples from healthy pregnant women were examined; 16.5% proved to be seronegative.

At the 76th meeting of the American Pediatric Society in April, 1966, KRUGMAN marked four milestones in the history of rubella research: (a) report by GREGG in 1941 on the teratogenic effect of the virus [1]; (b) isolation of rubella virus in tissue culture in 1962, independently by PARKMAN *et al.* in Washington [2] and by WELLER and NEVA in Boston [3]; (c) the 1965 rubella symposium in Philadelphia [4], where the experiences of the 1964 rubella epidemic in the U.S.A. and certain new aspects of symptomatology of the rubella syndrome had been discussed and summarized; (d) report by PARKMAN and MEYER in 1966 [5, 6] on the production of live attenuated vaccine from strain HPV-77 of rubella virus and on the results of an appropriately controlled field trial.

During the recent years further three teams have made attempts to produce appropriate vaccine from virus obtained in different tissue cultures [7].

In respect of the indication of active anti-rubella immunization, a survey of the population's immune status in various countries seems to be necessary with particular regard to women in the reproductive age. Data were made available on the incidence of rubella neutralizing antibodies by GIVAN *et al.* [8] and McLEAN *et al.* [9] from Canada, SEVER *et al.* [10, 11] from the U.S.A. and by OXFORD [12] from Sheffield.

Determination of the rubella neutralizing antibody has been performed by various methods [13, 14]. Of these chronologically the first was the inhi-

bition of enterovirus interference in primary green monkey kidney (GMK) cell cultures [8–11, 13]. Making use of appropriately adapted strains, the direct inhibition of cytopathic effect may reliably be measured in primary human amnion cultures [3], in RK-13 permanent rabbit kidney cells [12, 15, 16], in LLC-RK1 cells [17] and in SIRC cells [18]. A sensitive method for the quantitation of antibody is the haemadsorption-negative plaque method, too [19].

For the determination of anti-rubella immunity the haemagglutination-inhibition (HI) test [20] may also be used. This method has been developed during the last years and appears to yield results equivalent to the time- and work-consuming neutralization test.

To survey the immune status against rubella in Hungary, the neutralizing antibody content has been determined in a total of 1937 serum samples obtained from individuals 0 to 80 years old.

Materials and methods

Sera were collected during the period from November, 1966 to May, 1967.

Group I comprised a total of 1388 sera of which 1018 originated from samples taken for routine serology at the Health Insurance Institute, Budapest XII. Acute sera from 320 children with respiratory and nervous system diseases were provided by the clinical laboratory of this Hospital. Further 50 sera were obtained from young healthy adult males at recruitment for military service.

Group II. A total of 549 sera were obtained from healthy pregnant women at the II and XIV district antenatal clinics in Budapest. The pregnant women ranged in age from 17 to 35 years and the blood samples were taken in the 2nd to 8th months of pregnancy.

The sera were inactivated at 56°C for 30 minutes and stored at –20°C until used.

Neutralization test. Strain "Gilchrist" of rubella virus made available by the courtesy of Dr. J. SEVER (Bethesda, U.S.A.) was used as antigen. The strain has been adapted in this laboratory to RK-13 cells. The antigen was produced in Roux flask cultures of RK-13 cells and stored after titration at –20°C. The infectivity titre of the virus was checked every month and no significant decrease was observed during the period of storage. Twofold serial dilutions ranging from 1 : 4 to 1 : 128 were prepared from the sera and mixed each in an equal volume with virus containing 1000 TCD₅₀ per ml. Serum-virus mixtures were incubated overnight at +4°C. The serum dilution—virus mixtures were inoculated each into 3 tube cultures of RK-13 cells (0.1 ml per tube). The maintenance medium for the inoculated cultures consisted of PARKER's medium 199, containing 2% calf serum. The tubes were incubated in stationary position at 37°C and read on the 3rd and 5th days.

Results

Group I. Sex distribution of the donors of the 1388 sera was as follows: women, 821 (59%); men, 567 (41%). Rubella virus neutralizing antibody in serum dilutions 1 : 8 or higher was detected in 74% of the women and 72.5% of the men. In respect of positivity no significant difference having been found between women and men, the incidence of seropositivity in various age groups was evaluated regardless of sex (Table I).

Table I

Incidence of rubella virus neutralizing antibody in different age groups
Group I

Age group	Number of sera	Positive*	
		Number	per cent
0—11 months	46	24	52
1—5 years	102	44	43
6—10 years	112	56	50
11—15 years	98	57	58
16—20 years	84	62	74
21—30 years	213	182	85.5
31—40 years	131	115	88
41—50 years	168	147	87.5
51—60 years	198	160	81
61—70 years	165	134	81
>71 years	71	38	53.5
Total	1388	1019	73.4

* in dilutions $\geq 1:8$

In the age group 0 to 11 months, only 46 serum samples were examined. Thus it was not possible to evaluate the maternal immunity of 0 to 6 months old infants. In the relatively low (52%) seropositivity in this group, the disappearance of maternal antibodies may have played a role. Later on the rate of active immunity was found to increase parallel with age, exhibiting a steep rise in the age group 16 to 20 years, where the occurrence of antibodies attained 74%. Persons 21—30 years of age possessed rubella neutralizing antibodies in 85.5%, while 14.5% of the population was unprotected. The incidence of seronegative persons was 17.5% among females and 11% among males.

The percentage of seropositive persons was between 81% and 88%, the various age groups from 31 to 70 years. A decrease of positivity to 53.5% in the age group above 71 years was noted.

The neutralizing antibody titres obtained in the different age groups are shown in Fig. 1.

Neutralizing antibody titres obtained in RK-13 cells were low (1:18, 1:16). Their distribution was practically identical in all age groups from 0 to 80 years. Intermediate (1:32—1:64) and high ($\geq 1:128$) titres were encountered in 27.5%, 38%, and 54% of the age groups 16—20, 21—30 and 31—40 years, respectively. Above 40 years, the incidence of intermediate and high titres exhibited a decreasing tendency with values of 49%, 32%, 31.5% and 11% in the respective age groups.

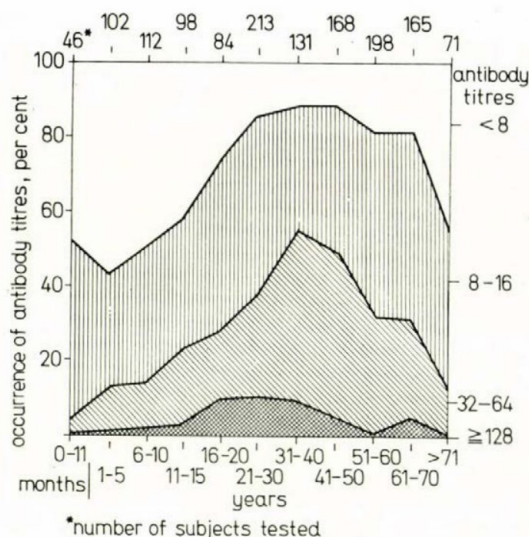


Fig. 1. Rubella virus neutralizing antibody titre levels in different age groups

Group II. The percentage of seronegativity in sera from pregnant was 16.5%. Low and high titres were found in 45.5 and 38%, respectively. Thus the serological data for pregnant were not different from those found in corresponding age groups of the general population.

Discussion

The neutralization test by inhibition of interference is too complicated for mass screening, and few authors have performed epidemiological studies by determination of neutralizing antibodies. In the present investigation, the antibody contents of sera were determined in RK-13 cell cultures in which the rubella virus produces focal cytopathic effect. This fact naturally involved the possibilities of subjective error in reading and evaluation. It is known that cell cultures contaminated with PPLO may exhibit focal lesions resembling those produced by rubella virus. To obviate this occasional source of error, 10 to 20 non-inoculated cultures were set up with each test. Initially, the final readings were made on the 7th and 10th days of incubation, but the most reproducible results were obtained by reading on the 5th day. Similarly, reading on the 5th day appeared to be optimal for the titration of immune sera prepared by DR. SEVER in monkeys and by ourselves in rabbits.

The results obtained in this laboratory are consistent with those of other authors. SEVER *et al.* [10, 11] demonstrated seropositivity in 35% of

children 1–10 years of age. In our material, this figure was 46.7% in the corresponding age group. Summing up the values found by OXFORD [12] in the age groups 1–4 and 5–10 years, seropositivity for the age group 1–10 was 46.9%. In our material, the incidence of seropositivity in the age group of 1–5 years was relatively high, 43%, as compared to the 21.4% found in Sheffield. The difference might be due to the fact that in Hungary many children are kept in daytime nurseries. This certainly involves an earlier exposure to rubella.

In the group from 11–15 years the seropositivity rates observed in Hungary, Canada and Sheffield are very similar, being 58, 60 and 68% respectively. The incidence of seronegativity in the age group 16–35 years also appears to be similar everywhere, ranging from 14 to 20%. Screening of pregnant women revealed 16.5% seronegativity, a value that would deserve consideration of active immunization of this group.

In a screening test performed by GIVAN *et al.* [8] two dilutions, 1 : 6 and 1 : 24 of each serum were examined by neutralization test. They observed a titre decrease in the age group of 25–40 years, followed later on by another rise. We have not observed this phenomenon.

In the future, more extensive application of the HI method is expected to allow a more efficient registration of rubella seropositivity all over the world with a better comparability of results obtained by identical technique.

Acknowledgements. For collection of sera, thanks are due Dr. HEDVIG HARMATH, Chief Physician (Health Insurance Institute, Maros utca, District XII, Budapest), Dr. E. CZEIZEL (National Institute of Public Health, Budapest) and Dr. MÁRTA SCHÄFFER (Blood Transfusion Station, Budapest X). The skilful technical assistance of Miss ERZSÉBET SCHLITZER and Miss ÉVA KUTASI is duly acknowledged. The author is indebted to Dr. I. DÖMÖK (National Institute of Public Health, Budapest) for valuable help and advice.

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IN VITRO SYNTHESIS OF ANTIBODY TO PHAGES OF MYCOBACTERIUM PHLEI

By

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Summary. Synthesis *in vitro* of antibody to phages of *M. phlei* has been studied. Spleen cell suspensions of rabbits immunized with phages active against *M. phlei* gave a neutralizing antiphage antibody *in vitro* in the presence of the phages. This phenomenon was partly inhibited by actinomycin D.

Contrary to the detailed studies of the synthesis of antibodies to coli phages, there are no data available regarding the synthesis *in vitro* of antibodies to mycophages. The synthesis *in vitro* of antibody and its inhibition by actinomycin D, active against phages of *Mycobacterium phlei*, has therefore been studied using cell suspensions of immunized rabbits.

Materials and methods

The phage used as antigen in the experiments was isolated from soil by enrichment [1], by the method of HAUDUROY and GROSSET [2]. This monovalent specific phage was active only against *M. phlei*. The phage suspension was filtered through a G5 glass filter then purified by differential centrifugation as follows: after centrifugation at 4000 *g* for 20 minutes the supernatant was centrifuged at 35,000 *g* for 30 minutes and the phage-pellets were resuspended in isotonic sodium chloride solution. The phage suspension gave a uniform picture at analytical centrifugation and its titre proved to be $10^{9.7}$ – $10^{10.7}$ /ml by the agar layer method [3].

Rabbits were immunized with the phage suspension without adjuvant four times, as follows: (i) intramuscularly (4 ml); (ii) after two weeks subcutaneously (4 ml); (iii) one week later intramuscularly (2 ml), and into the foot-pad (2 ml); and (iv) one week later intravenously (2 ml) and into the footpad (2 ml).

Antibody titres present in the sera were expressed by the “*k*” value of the sera measured by the reaction of phage-neutralization of the sera of the immunized rabbits

$$k = \frac{2.3 \cdot D}{t} \log \frac{p_0}{p_t}$$

where p_0 and p_t are the number of phages at zero and t min, respectively, and D represents the dilution of the sera [4].

The rabbits were killed one week after the fourth immunization and a cell suspension of the spleens was prepared [6] in EAGLE’s minimal essential medium [5]. The cell suspension was washed and after counting the cells a dilution of 10^7 spleen cells/ml was prepared in EAGLE’s medium.

The following three *in vitro* systems were prepared: (1) spleen cell suspension; (2) spleen cell suspension + phages active against *M. phlei* in a final concentration of 10^6 /ml; (3) spleen cell suspension + phages active against *M. phlei* in a final concentration of 10^6 /ml + actinomycin D in a final concentration of 10 μ g/ml. These systems were incubated at 37°C.

Samples were obtained at intervals indicated in Fig. 1. All the samples were centrifuged and after adding *M. phlei* phage also to the supernatants of the samples of system (1) in a final concentration of 10^6 /ml, the titre of the "viable" phages (not neutralized *in vitro*) of the supernatants was measured by the agar layer method. This titre reflects the quantity of antibodies present in the system.

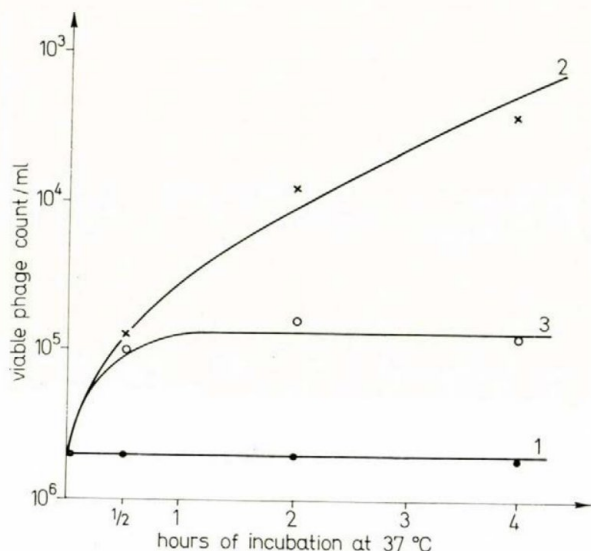


Fig. 1. Synthesis *in vitro* of antiphage-antibody by spleen cells of rabbits immunized with phages active against *M. phlei*. The curves show the count of "viable" phages in the three systems used. The systems (1, 2, 3) are given in the text

Results

The k values of the sera proved to be zero before the first injection, $4-5 \text{ min}^{-1}$ before the fourth immunization and $5-6.5 \text{ min}^{-1}$ one week after the fourth injection and remained zero in the control (not immunized) animals.

Fig. 1 clearly shows that the spleen cell suspensions of rabbits immunized with phages active against *M. phlei* yielded a neutralizing antibody *in vitro* but only in the presence of phages. The number of viable phages diminished at a highly increased rate (curve 2). In system (1), where the spleen cells had not incubated with phages but these were added to the supernatants obtained by centrifuging the cells, no measurable antiphage-titre could be observed (curve 1). In the presence of actinomycin D the number of "viable" phages diminished at the beginning of the experiment, just as in system (2). Thereafter, no further decrease could be observed in contrary with the system without actinomycin D (curve 3).

The points in Fig. 1 are representing average values of three experiments

Discussion

In the systems described above an *in vitro* booster effect has been studied. Spleen cells of rabbits immunized with phages active against *M. phlei* were incubated with the same phages with and without actinomycin D. The number of "viable" phages diminished under the conditions employed, but this diminution was significantly minor in the presence of actinomycin D. It may be concluded that the diminution of the number of "viable" phages was due to neutralizing antibodies. As the phages are rapidly neutralized, it may be surmised that stimulation by phages liberates an antibody-pool of the sensitized spleen cells (release mechanism). The phage-neutralizing effect of the spleen cells of the sensitized rabbits ceased after 30 minutes incubation with actinomycin D. As actinomycin D inhibits protein synthesis *de novo*, and in system (2), to which no actinomycin had been added, a further decrease occurred in the number of "viable" phages, it may also be assumed that *de novo* antibody synthesis, too, had a role in the neutralizing effect. This could be proved by experiments with radioactive isotopes.

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EFFICIENCY OF SMALLPOX REVACCINATIONS REPEATED AT THREE YEAR INTERVALS

I. FOLLOW-UP OF THE IMMUNOLOGICAL STATUS OF THE STAFF OF AN INFECTIOUS DISEASE HOSPITAL

By

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Summary. The reaction to smallpox revaccination in persons who had been successfully revaccinated 3 years earlier has been studied.

Immediately before the actual revaccination, 32.8% and 12.0% of the persons studied had no detectable haemagglutination-inhibiting (HI) and neutralizing antibodies, respectively. Major reaction was produced by 47.6%, equivocal reaction by 52.4% of the subjects. Equivocal reaction healed with and without crust in 23.8% and 28.6%, respectively.

An HI antibody response was given by the vaccinees producing major reaction almost regularly, by those producing equivocal reaction, exceptionally. Neutralizing antibody was produced by all the vaccinees giving major reaction and also by those whose equivocal reaction had healed with a crust. For this serological reason, it seems justified to distinguish two types of equivocal reaction, *viz.*, equivocal-1, healing with a crust, and equivocal-2, healing without crust. It is proposed that the equivocal-1 reaction should be considered indicative of doubtful success, while equivocal-2 indicative of unsuccessful revaccination.

Neutralizing antibody response was detectable by the end of the first postvaccination week in 47% of the cases.

In accordance with the recommendation by the WHO [1], in Hungary persons at risk of exposure to smallpox should be revaccinated every third year. For this reason a number of the employees of the Central Hospital for Infectious Diseases who had been revaccinated in 1963 were revaccinated in 1966.

On this occasion we wished to obtain information on the residual immunity and on the efficiency of the present revaccination. For this purpose we studied the distribution of the cutaneous reactions and the pre- and post-revaccination serological titres. Furthermore, the correlations between pre-vaccination titres and the frequency of major reaction and between the type of the local reaction and the antibody response have been studied.

Materials and methods

Revaccination. Hospital employees of both sexes from 21 to 60 years of age were revaccinated. They had been vaccinated and revaccinated in infancy and at the age of 6–10 years, respectively, and revaccinated again in 1963, when each of them produced a major reaction. All previous vaccinations were performed with a dermovaccine prepared from the "Budapest"

vaccine strain, the strain exclusively used in Hungary. The present vaccinations were performed on the upper arm. Two scarifications were made at a distance of about 10 cm. One of these was a single scarification 4 mm in length, the other consisted of 4 scarifications, each 4 mm in length in the form of a double cross. All the vaccinations were performed by the same person highly experienced in vaccination.

Vaccines. Two kinds of vaccine were used. Both had been prepared from calf lymph in the Institute for Serobacterial Production and Research "Human", Budapest, by Dr. J. SZATHMÁRY, one from the international vaccine strain Elstree, the other from the Budapest strain. The latter strain, received from Professor H. A. GINS (Berlin) in 1927, has been maintained in continuous alternate calf-rabbit passages. The titre of the Budapest strain vaccine was 1.38×10^8 PFU, that of the Elstree strain vaccine was 1.03×10^8 PFU per ml, as determined on chick embryo chorioallantoic membrane (CAM). Thirty-five persons were vaccinated with the Budapest vaccine, 28 with the Elstree vaccine.

Cutaneous reactions were read 3 and 7 days after vaccination. Evaluation was based on the second reading, according to the recommendation of the WHO [1]. The vaccinations producing major reaction were accepted as successful. The reactions "equivocal" according to the WHO recommendation, were, however, divided into two groups: (1) the term equivocal-1 reaction means that on the 7th day the papule seen 3 days after vaccination was covered by a crust; (2) the term equivocal-2 reaction means that the 3-day papule had healed without crust by the 7th day. Equivocal-1 reaction was considered as immunologically equivocal, equivocal-2 reaction as unsuccessful. Revaccination producing no reaction did not occur in the group under study. Each site of scarification was qualified separately, i.e., two reactions were qualified for each person. The result of vaccination for subject was qualified on the basis of the stronger reaction which developed as a rule at the site of the double-cross scarification. Accordingly, persons were grouped on the basis of their cutaneous reaction as follows. "Take": at least one of the scarification sites exhibited a major reaction. "Equivocal": at least one of the scarification sites exhibited an equivocal-1 reaction, the other an equivocal-1 or equivocal-2 reaction. "Unsuccessful": equivocal-2 reaction at both scarification sites.

In the present paper we did not separate the groups vaccinated with the two different vaccines.

Serum samples. Blood was taken immediately before vaccination, and 1 week and 3 weeks after vaccination. Sera were inactivated at 56°C for 30 minutes and stored in the frozen state.

Neutralization tests were carried out on CAM. Serial fourfold dilutions of serum were tested and the dilution causing 60% pock reduction was accepted as titre. The test has been described in detail elsewhere [2].

Haemagglutination-inhibition (HI) test was performed by TAKÁTSY's [3] Microtitrator technique as modified by SZATHMÁRY [4].

Statistical analysis of the results of antibody measurements was based on the $-\log_2$ value of titre. To judge deviations between geometric means deriving from measurements independent of each other, STUDENT's two-sample *t* test, in the follow-up studies of titres STUDENT's one-sample test, were employed. For judging variances, FISHER's test was used. Classificatory data were analysed by the χ^2 test. In evaluating the differences $p < 5\%$ was considered the fiducial limit.

Results

Distribution of revaccination results. On the basis of the criteria under Materials and methods, out of the 63 persons under study 30 were classified into the group "take", 15 into the group "equivocal" and 18 into the group "unsuccessful".

Serological tests. (a) Pre- and post-vaccination neutralization titres.

a(1) Neutralization test was performed in 58 prevaccination samples (Fig. 1). The geometric mean of titres was 6.8, 5.2 and 4.3 in the groups "take", "equivocal", and "unsuccessful", respectively. The differences were not significant (Table I). Fig. 1 shows that 7 subjects (12.0%) had no detectable neutralizing antibody. It is also clear that a major reaction had developed

even in spite of a prevaccination antibody titre as high as 1:64, i.e., the highest prevaccination titre found in the present study. Three of the four subjects with such a high titre produced major reaction, the fourth developed an equivocal-1 reaction.

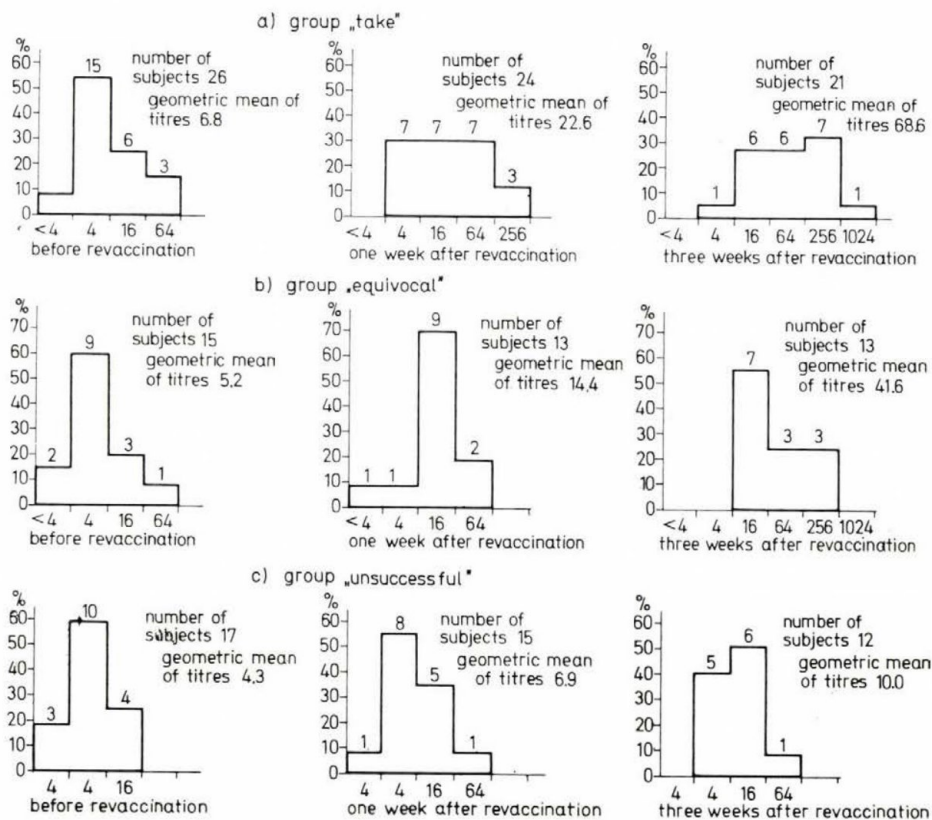


Fig. 1. Distribution of neutralization titres

Table I

Comparison of the average neutralizing antibody titres of persons revaccinated with different results

Group	Before revaccination			One week			Three weeks		
				after revaccination					
	$\bar{x}_1 - \bar{x}_2$	t_x	$p\%$	$\bar{x}_1 - \bar{x}_2$	t_x	$p\%$	$\bar{x}_1 - \bar{x}_2$	t_x	$p\%$
Take—equivocal	0.37	$t_{39} = 0.384$	> 60	0.65	$t_{35} = 1.264$	> 20	0.72	$t_{32} = 1.055$	> 30
Take — unsuccessful	0.65	$t_{41} = 1.324$	> 10	1.7	$t_{37} = 2.677$	< 2	2.67	$t_{31} = 4.049$	< 0.1
Equivocal — unsuccessful	0.28	$t_{30} = 0.306$	> 70	1.05	$t_{26} = 1.860$	> 5	2.05	$t_{23} = 3.349$	< 1

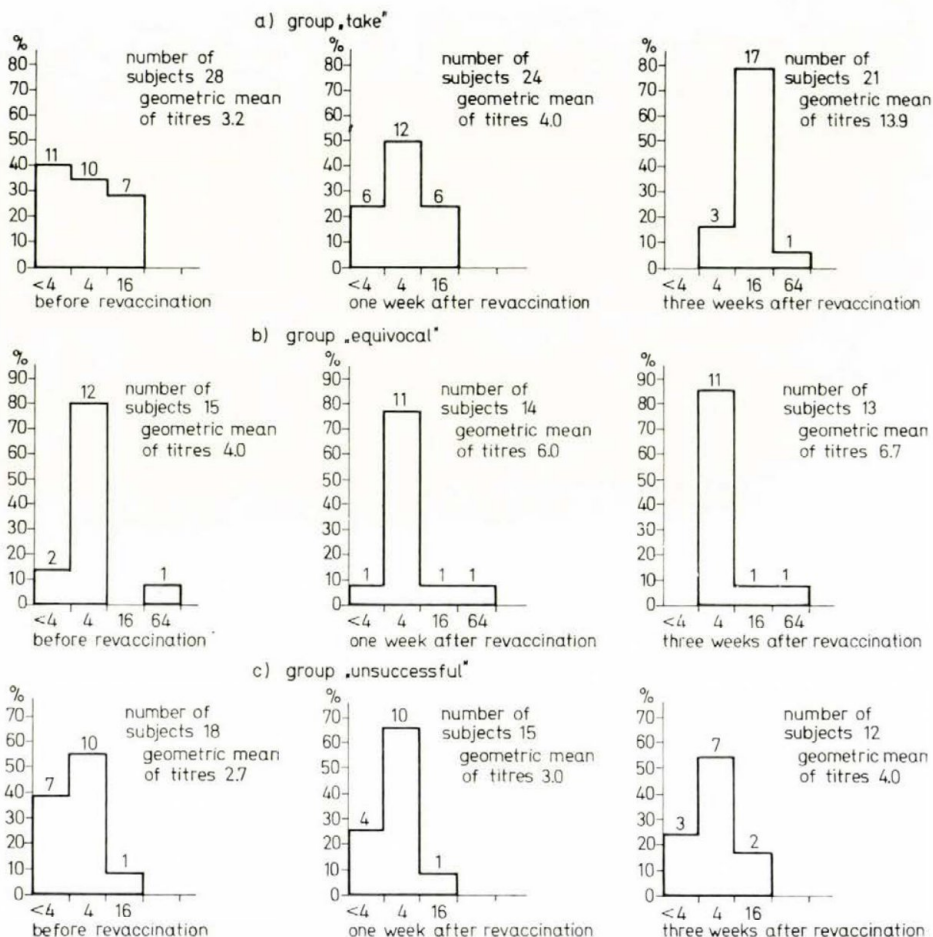


Fig. 2. Distribution of HI titres

a(2) One-week postvaccination sample was available for the neutralization test from 52 vaccinees (Fig. 1). The geometric mean of titres for the groups “take”, “equivocal” and “unsuccessful” was 22.6, 14.6 and 6.9, respectively. The difference between the two extreme values was significant, the other two differences were not significant statistically (Table I). Furthermore, the difference between the geometric mean of the one-week postvaccination titres and that of the prevaccination titres, was significant for the group “take” but not for the other two groups (Table II). Fig. 3 shows that one week after vaccination no neutralization titre higher than 1 : 64 occurred in the groups “equivocal” and “unsuccessful”.

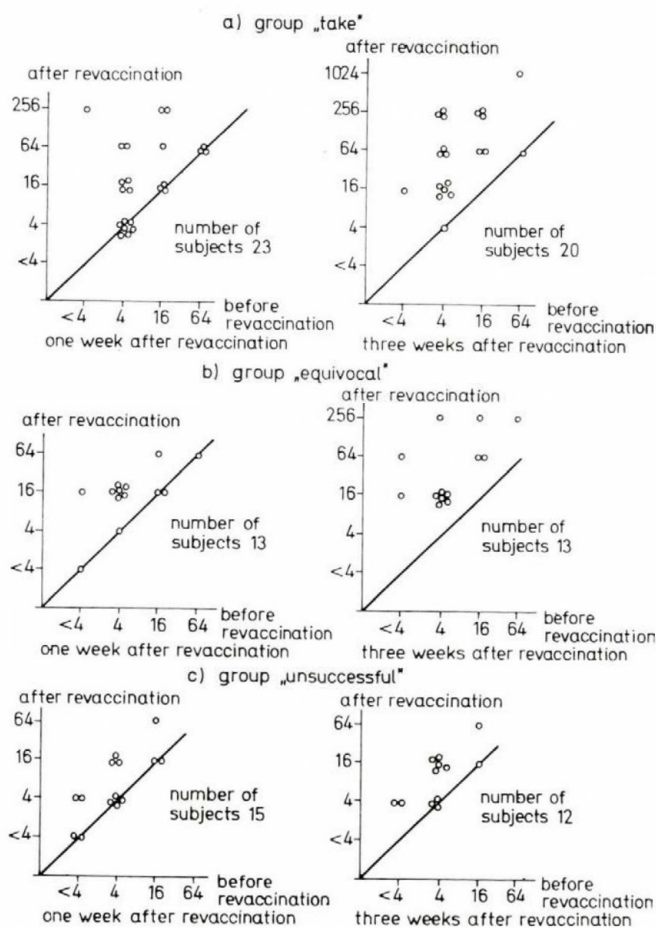


Fig. 3. Comparison of pre- and postrevaccination neutralization titres

Table II

Average neutralization titres in the function of time

Result of revaccination	Before revac- cination	One week			Three weeks		
		after revaccination					
		$\frac{\Sigma x_1 - \Sigma x_2}{N}$	t_x	$P\%$	$\frac{\Sigma x_1 - \Sigma x_2}{N}$	t_x	$P\%$
Take	2.66	1.42	$t_{20} = 2.529$	< 5	3.42	$t_{20} = 3.174$	< 1
Equivocal	2.50	1.33	$t_{11} = 1.768$	> 10	3.00	$t_{11} = 2.570$	< 5
Unsuccessful	1.83	0.50	$t_{11} = 0.997$	> 30	1.16	$t_{11} = 1.788$	> 10

As to the frequency of fourfold or greater rises in neutralization titre, there was no significant difference between the three groups. Fig. 3 shows that in the group "take" 10 of the 23, in the group "equivocal" 8 of the 13, in the group "unsuccessful" 6 of the 15 subjects developed such a rise in titre. This means that there was no correlation between the cutaneous reaction and the promptness of the antibody response.

a(3) Three-week postvaccination sample was available for neutralization test from 46 vaccinees. The geometric means were 68.6, 41.6 and 10.0 for the "take", "equivocal" and "unsuccessful" groups, respectively. The values for the group "unsuccessful" were significantly lower than those for the other two groups (Table I). Accordingly, the geometric means of the three-week postvaccination titres for the first two groups were significantly higher than the corresponding prevaccination titres (Table II).

Table III

Comparison of the average HI titres of persons revaccinated with different results

Group	Before revaccination			One week			Three weeks		
	$\bar{x}_1 - \bar{x}_2$	t_x	$P\%$	after revaccination					
				$\bar{x}_1 - \bar{x}_2$	t_x	$P\%$	$x_1 - \bar{x}_2$	t_x	$P\%$
Take—equivocal	0.29	$t_{41} = 0.845$	> 30	0.28	$t_{36} = 0.593$	> 50	1.34	$t_{32} = 3.754$	< 0.1
Take—unsuccessful	0.38	$t_{44} = 0.702$	> 40	0.40	$t_{37} = 0.911$	> 30	1.97	$t_{31} = 5.129$	< 0.1
Equivocal—unsuccessful	0.77	$t_{31} = 1.251$	> 20	0.68	$t_{27} = 1.486$	> 10	0.63	$t_{23} = 1.240$	> 20

(b) *Prevaccination and postvaccination HI titres.*

b(1) Prevaccination HI titres were determined in 61 sera (Fig. 2). The geometric means were 3.2, 4.0 and 2.7. The differences were not significant (Table III). Fig. 2 shows that in the sera of 20 persons (32.8%) prevaccination HI antibody was not detectable. Like the prevaccination neutralization

Table IV

HI titres in the function of time

Result of revaccination	Before revac- cination	One week			Three weeks		
		after revaccination					
		$\frac{\Sigma x_1 - \Sigma x_2}{N}$	t_x	$P\%$	$\frac{\Sigma x_1 - \Sigma x_2}{N}$	t_x	$P\%$
Take	2.00	0.10	$t_{19} = 0.570$	> 50	2.00	$t_{19} = 11.370$	< 0.1
Equivocal	2.16	0.16	$t_{11} = 0.476$	> 60	0.32	$t_{11} = 0.847$	> 40
Unsuccessful	1.27	0.18	$t_{10} = 0.989$	> 30	0.36	$t_{10} = 1.475$	> 10

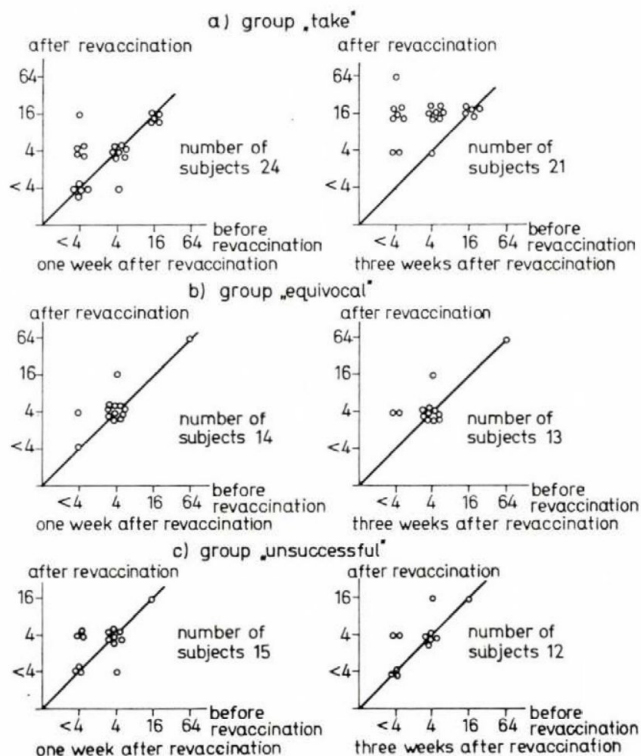


Fig. 4. Comparison of pre- and postrevaccination HI titres

titres, the prevaccination HI titres were unsuitable to predict the result of vaccination. Subjects with a prevaccination titre of 1 : 16 were vaccinated with success, whereas the vaccination of some of those devoid of HI antibodies remained unsuccessful.

b(2) HI antibodies were determined in 53 one-week postvaccination samples (Fig. 2); the geometric means were 4.0, 6.0 and 3.0. There was no significant difference between these values and none of these differed from the geometric mean of the corresponding prevaccination titres (Tables III and IV).

Fourfold or greater rise in HI titre was developed by the end of the first week by 5 of the 24 persons in the group “take”, by 2 of the 14 in the group “equivocal”, and by 3 of the 15 in the group “unsuccessful”. Accordingly, the HI antibody response was slower than the neutralizing antibody response in all the three groups. Furthermore, there was no correlation between the type of the cutaneous reaction and the promptness of the antibody response. From the one-week postvaccination titres the lowest HI antibody level to be considered characteristic of successful vaccination could not be determined.

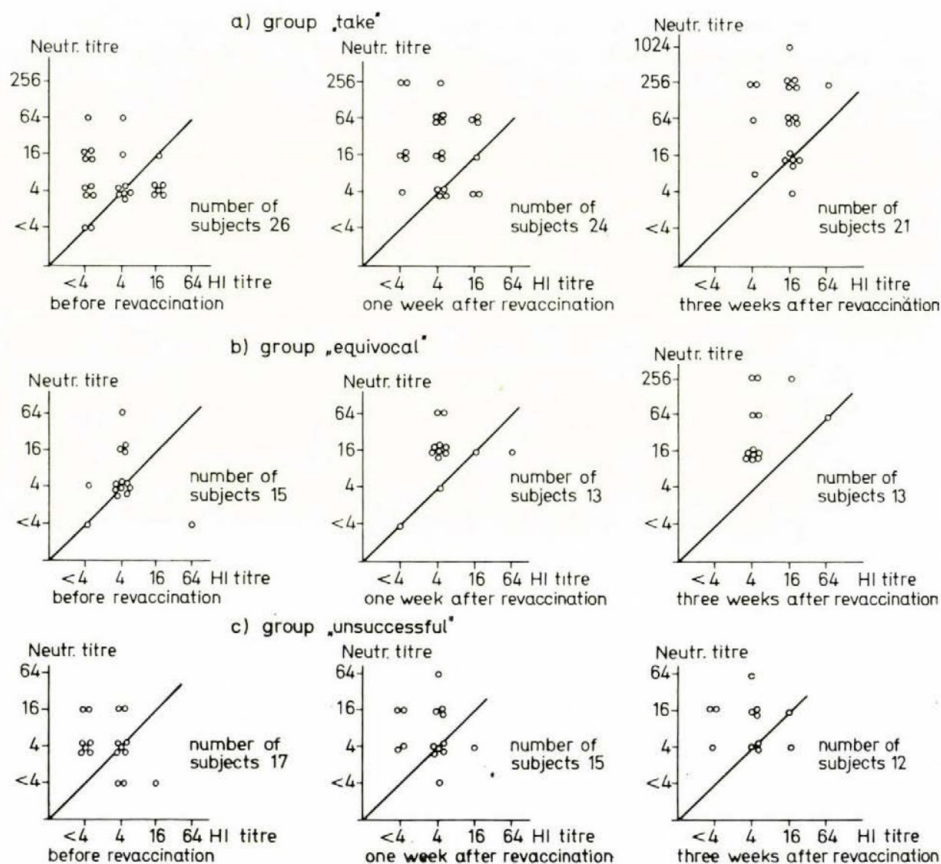


Fig. 5. Correlation between neutralizing and HI antibody titres

b(3) The HI antibody titre was determined in 46 samples taken 3 weeks after vaccination (Fig. 2). The geometric means of titres were 13.9, 6.7 and 4.0. This meant a significantly higher geometric mean for the group "take" than for the other two categories. Accordingly, the rise in HI titre was significant only for the group "take".

Fig. 4 shows that in the group "take" 15 of the 21 vaccinees developed fourfold or greater HI antibody response. Among the remaining 6 vaccinees, whose titres showed no increase, 5 had a relatively high prevaccination HI titre: 1 : 16. In each of the groups "equivocal" and "unsuccessful" only three persons had developed a fourfold or higher HI antibody response by the end of the 3rd week. Accordingly, the cutaneous reaction was in a closer correlation with the HI antibody response than with the neutralizing antibody response. It seemed, however, impossible to tell which HI antibody level was to be considered characteristic of take.

(c) *Correlation between HI and neutralization titres.* As shown in Fig. 5, in the majority of the prevaccination samples the neutralization titres were higher than the HI titres and this difference became more pronounced one week after vaccination and still more pronounced in the three-week samples. This tendency appeared to be independent of the success of vaccination.

Discussion

In the present study persons revaccinated with two different vaccines are treated together. This is justified, for the vaccinees were grouped according to the type of cutaneous reaction. Principally, a major reaction secures protection independently of the vaccine strain used, provided the vaccine is meeting requirements. Preliminary calculation has shown that in the present material the geometric means had not been influenced significantly by the quality of the vaccine strain. Only one difference was significant in this respect: the geometric mean of the three-week postvaccination HI titres was higher for those vaccinated with the Budapest strain than for those vaccinated with the reference strain. However, this difference is of no importance as regards the following conclusions.

The subjects under study, being at potential risk, are revaccinated every third year. It would be of interest to know (i) whether or not a person to be vaccinated is susceptible to smallpox; (ii) whether or not the protection has been reinforced by the revaccination. The correct answers to these questions can be approached but indirectly, *viz.* by analyzing the cutaneous reactions and serological data.

The cutaneous reaction, though giving no information as to whether a person needs revaccination, is of great value in the retrospective analysis. It is generally believed that a major reaction to revaccination means a lack of protection against smallpox. However, KAPLAN [5] emphasized that revaccination with the usual dose of virus is a too severe challenge. The frequency of major reaction may be decreased by reducing the introduced amount of virus. Our experience that subjects who developed major reaction to double-cross scarification often showed equivocal-2 reaction at the site of the single scarification is consistent with KAPLAN's inference. As to the success of revaccination it is the most general and least risky assumption that immunity to smallpox is not reinforced unless a major reaction is developed.

Several authors [6, 7, 8] have used serological data for evaluating the level of protection and the efficiency of revaccination. That serum antibodies play a considerable role in the immunity against smallpox is underlined by the successful use of gamma globulin in smallpox prophylaxis and therapy [9, 10]. However, little is known about the antibody level necessary to solid protection.

In accordance with the above considerations, lack of protection might be indicated by (i) lack of prevaccination neutralizing antibodies or by (ii) the major reaction given to revaccination. Accordingly, 12.0% and 47.6%, respectively, of the subjects under study should be considered unprotected as early as three years after the previous revaccination (Fig. 1). These data are as inconsistent as the usual indicators of the success of revaccinations. On the basis of the takes 47.6%, on that of a fourfold or greater rise in the neutralizing antibody level 86.6%, of the revaccinations should be considered successful (Fig. 3).

From the practical point of view the following should be taken into consideration.

(a) Three years after a successful revaccination about half of the revaccinations were successful. This is not an extreme value. CROSS [8] reported on 77% takes in a population vaccinated 2—5 years before. Thus, accepting the revaccination challenge as to reflecting the immunological status correctly would mean that vaccinations at three-year intervals fail to secure continuous protection. Such an interpretation would, however, be inconsistent with the common experience. Thus, the reaction to a revaccination with potent vaccine does not correctly reflect the prevaccination immunological status.

(b) We have succeeded in dividing into two well-defined groups the reactions qualified equivocal according to the WHO recommendation. In one of these groups (equivocal-1) the reaction healed with a crust, in the other (equivocal-2), without crustation. Serological tests have shown that in these two groups the antigenic stimulus was also different. In the case of the equivocal-1 reaction the neutralization titre increase was consistent, whereas the equivocal-2 reaction was accompanied by a minimal, if any, reaction. The HI titre showed no rise in these groups, suggesting that there was no significant virus multiplication.

(c) Estimation of the immunizing effect of the equivocal reactions on the basis of serological findings has been hindered by contradictory data. Some authors [11, 12] suggest that all revaccinations inducing a cutaneous reaction are accompanied by an antibody response, while others [6, 13, 14] found an inconsistent antibody response in such cases. The contradiction might be attributed to differences in the prevaccination immunological status, in the quality of the vaccine and/or in the vaccination technique. Accordingly, we can only predict that in persons who were primovaccinated in infancy and revaccinated three years ago, a repeated revaccination resulting in an equivocal-1 reaction will be followed by a neutralizing antibody response.

(d) Three years after a successful vaccination the cutaneous reaction to a repeated revaccination cannot be predicted from the prevaccination HI and neutralizing antibody titres.

(e) It is the increase in the HI titre that fits best with the type of the cutaneous reaction.

(f) Of the 21 major reactions 6 were not followed by an appreciable HI titre rise, suggesting that major reactions may not indicate virus multiplication. According to EHRENGUT's [15] experience even pustules may be produced on an allergic basis.

(g) Ten out of the 23 vaccinees in the group "take" and 14 out of the 28 in the groups "equivocal" and "unsuccessful" had produced an appreciable neutralizing antibody response by the end of the first week. Thus, the booster effect, as measured serologically, was poor. It might be noted in this respect that the protecting effect of a revaccination after exposure to smallpox infection is not quite clear. According to LEAKE [16] revaccination on the day of exposure results in complete protection, whereas RICKETTS (cit. [7]) found great individual variations. In this respect the data presented by BAUER *et al.* [17] in connection with the Madras field trial are also inconclusive. Thus, further experience is needed to evaluate the protective effect of the revaccination of contact persons.

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COMPARISON OF INTERFERON PRODUCTION IN VITRO BY LEUKOCYTES FROM HEALTHY AND POLYCYTHAEMIC PERSONS

By

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Summary. Interferon production *in vitro* by leukocytes from healthy and polycythaemic persons has been compared. As inducers parainfluenza type 1 (Sendai) and Newcastle disease (ND) viruses were used. Interferon was titrated with vesicular stomatitis virus in permanent human amnion cell cultures. The interferon producing capacity of leukocytes from patients with polycythaemia vera was significantly lower as compared to leukocytes from healthy donors. The average interferon titre of polycythaemic and normal persons was 50 and 220, respectively. As referred to the average control titre values, the interferon titres produced by leukocytes of 18 out of 21 patients (85%) was significantly lower, whereas that of the remaining 3 persons (15%), similar. Results are discussed in comparison to the enhanced interferon production by leukocytes from patients with myeloid leukaemia.

In some previous studies we have found increased interferon production by leukocytes from patients with myeloid leukaemia as compared to normal controls [1]. LEE *et al.* [2, 3] reported decreased or unchanged production of interferon by leukocytes obtained from persons with lymphoid leukaemia. It seemed to be of interest to perform similar studies on leukocytes of patients with polycythaemia vera.

Materials and methods

Ill and healthy donors. Leukocytes obtained from a total of 21 patients were used. Prior to blood sampling each patient was repeatedly subjected to extensive clinical examination. The patients were divided into two groups on the basis of the history, results of physical examination, erythrocyte, leukocyte and platelet counts, haematocrit readings, haemoglobin content, differential count in peripheral blood and bone marrow. Group I comprised patients in the phase of exacerbation, whereas group II those in the phase of remission. On the day of blood sampling and on the day before no treatment was applied. Several months prior to this study part of the patients underwent treatment with ^{32}P . As controls, blood samples from healthy untreated persons were used.

Preparation of purified leukocyte suspensions. The method suggested by STRANDER and CANTELL [4] was employed. Freshly withdrawn citrated blood was centrifuged, the plasma removed and to the suspension 5% EDTA solution was added at a ratio of 10 : 1 v/v. Haemolysis was induced by the addition of 8 volumes of 0.83% ammonium chloride solution. The above mixture was incubated for 10 minutes at $+4^{\circ}\text{C}$ and centrifuged. The supernatant was discarded and the sediment treated once more as described above. The leukocytes finally obtained were suspended in the medium required.

Viruses. Parainfluenza type 1 virus (strain Sendai) and NDV "H" strain were maintained by serial passage in the allantoic cavity of embryonated hen's eggs. Virus suspensions were stored at $+4^{\circ}\text{C}$ until used.

The Indiana strain of vesicular stomatitis virus (VSV) was maintained by passages in HeLa cells. The virus suspensions were stored at -70°C until used.

Tissue culture. The "U" line of permanent human amnion cell culture (obtained by the courtesy of Dr. K. CANTELL, State Serum Institute, Helsinki, Finland) was cultivated in a medium consisting of PARKER's 199 with 10% calf serum and 0.125% sodium bicarbonate.

Production of interferon. Washed leukocytes were suspended in PARKER's 199 with 10% calf serum and 0.25% sodium bicarbonate pH 7.4, and infected with Sendai or ND virus. The infected leukocyte suspension was incubated at 37°C in rotated siliconed centrifugal tubes tightly closed with rubber stoppers. The roller drum was rotated at 18 r.p.m. The density of suspended cells was $5 \times 10^6/\text{ml}$ and the input multiplicity of virus was 100. The total volume of the system was 5 ml. The incubation period was 24 hours.

Determination of interferon titre. At the end of the incubation period the leukocyte suspension was centrifuged and the supernatant dialysed for 72 hours against 100 volumes of pH 2.0 glycine-HCl buffer. The pH was then readjusted to 7.4 by dialysation against phosphate-buffered saline. From the preparations thus obtained, fourfold dilution series were prepared in Melnick-Earle solution containing 2% calf serum, 0.5% lactalbumin hydrolysate and 0.125% sodium bicarbonate with penicillin and streptomycin. One ml of each dilution step was added to tubes in duplicate of monolayer cultures of U cells. The tubes were incubated overnight, washed twice with phosphate-buffered saline and infected with 50 CPD₅₀ of VSV in 1 ml of the above medium. Final reading was made at the time when the control interferon-free cultures exhibited 70–100% cytopathic destruction. The dilution of interferon ensuring the protection of 50% of the cells was considered the unit titre. The limit of error of this procedure was $\pm 50\%$.

Results

In Table I are shown the titres of interferon induced by Sendai or ND viruses in purified leukocyte suspensions obtained from 21 polycythaemic and 7 healthy persons. Data concerning the preceding treatment and the actual phase of the disease are also included.

In the majority of patients with polycythaemia vera the interferon producing capacity of the leukocytes decreased as compared to the controls. Leukocytes of 14 out of the 21 patients (66%) produced considerably less interferon, while the 7 others (33%) amounted comparable to those of controls. Considering the totality of the results with both inducer viruses, the average control interferon titre was 220, whereas that of the polycythaemic leukocytes 50. The difference was more than fourfold. In comparison to the mean control titres, the incidence of decreased titres was 85% (18 patients), while in 15% (3 patients) similar to those of the controls were encountered.

No correlation was found between the applied treatment, the phase of the disease (exacerbation or remission) and the interferon production by leukocytes.

Discussion

The reduced interferon production by leukocytes from polycythaemic patients is in sharp contrast to the opposite activity of the same cells from patients with myeloid leukaemia. This fact seems to be important from several respects. It has been known that polycythaemia vera is characterized by

Table I

Comparison of interferon production by leukocytes from healthy and polycythaemic patients

Polycythaemic donors	Healthy donors (controls)	Beginning of the disease (year)	Number of preceding ^{32}P treatments	Stage of polycythaemia	Interferon titre	
					Sendai	NDV
O. J.	K. S.	1967	—	E	< 4	4
					64	64
M. F.	T. J.	1967	—	E	< 4	n. e.
					256	n. e.
Sz. K.	Sz. A.	1964	—	E	8	4
Sz. J.		1967	—	E	4	n. e.
					128	256
B. J.		1967	—	E	16	n. e.
Zs. I.		1957	3	E	4	n. e.
A. S.		1963	2	E	32	n. e.
B. A.		1961	3	E	16	n. e.
	H. Gy.				32	n. e.
B. A.		1961	3	E	16	n. e.
B. B.		1963	2	E	16	n. e.
V. J.		1956	4	E	32	n. e.
K. P.		1959	2	E	16	n. e.
B. V.		1960	1	E	64	n. e.
					256	n. e.
Sz. G.		1960	3	E	256	n. e.
Cz. M.		1959	4	E	64	n. e.
M. M.		1956	4	R	128	n. e.
					128	n. e.
O. J.		1961	3	E	32	16
M. L.		1964	1	R	64	64
Ü. L.		1957	3	R	64	64
O. J.		1959	2	R	64	64
	M. J.				512	512

E = stage of exacerbation

R = stage of remission

n. e. = not examined

enhanced myelopoietic activity, increased leukocyte count and shift to the left of the differential count. Thus it may be stated that the reduced interferon producing capacity of leukocytes from polycythaemic patients proves that the enhanced interferon production by leukocytes from patients with myeloid leukaemia cannot be explained by the stimulation of leukocyte pro-

duction and by the shift to the left of the differential count. (It should be remembered that polycythaemia may be considered a potential pre-leukaemic condition, developing into true leukaemia in part of the patients [5].) Some recent electron microscopic studies and relevant epidemiological data are suggestive of the possibility of a viral aetiology of human leukaemias [6]. It is known that interferon is a natural non-specific anti-viral protective factor of the organism [7, 8]. Considering certain animal experiments demonstrating that some carcinogens such as urethane, 3-methyl-cholanthrene, etc. are inhibiting interferon production [9, 10], we suppose that in the human organism substances inhibiting the production of interferon may facilitate the action of a virus possibly responsible for leukaemic transformation. In this respect the reduced interferon production by leukocytes in the majority of polycythaemic patients may have some significance.

Further studies are in progress to elucidate the cause and mechanism of the phenomenon.

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EXAMINATION IN VITRO OF THE VIRUS INHIBITORY ACTION OF A NEW BENZTHIAZOLE DERIVATIVE

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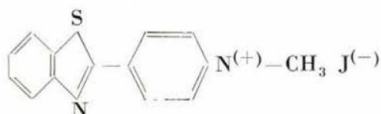
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Summary. The growth inhibitory action on various myxovirus strains of a new benzthiazole derivative [2-(4'-pyridyl)benzthiazole monomethyl iodide] has been examined. The compound was found to inhibit the growth of influenza A-2 (Singapore 4/57) strain in surviving chorioallantoic membrane cultures. It was neither virucidal nor did it inhibit virus adsorption. It interfered with some process in the early intracellular phase of the viral cycle.

TAMM and EGGERS [1] reported certain benzimidazole derivatives to inhibit the growth of some myxo- and picorna-viruses. The synthesis of certain benzthiazole derivatives has been recently described [2]. The compounds were examined as to their inhibitory action on various myxoviruses and the correlation of their activity and chemical structure [3]. The present paper reports on results of further examinations of the action of 2-(4'-pyridyl)benzthiazole monomethyl iodide, the benzthiazole derivative found the most active among those available.

Materials and methods

The compound was synthesized by K. HIDEG and O. H. HANKOVSKY, Institute of Pharmacology, University Medical School, Pécs, and kindly supplied to us for examination. Designation: H-253. Molecular weight: 354.22. Melting point: 277–280°C. Dissolves readily in water. Structural formula is as follows



Tissue cultures and media. Surviving chorioallantoic membrane (CAM) fragments were maintained in suspension in glucosol medium containing phosphate buffer and egg white [4]. Human embryonic fibroblast cultures obtained by trypsinization of embryos and a persistent line "U" of human amnion cells obtained by the courtesy of Dr. K. CANTELL, State Serum Institute, Helsinki, Finland, were used. The cultures were grown and maintained in PARKER's 199 medium containing 10% and 2% calf serum, respectively.

Viruses. The following strains were used: Influenza A-0 subtype strain PR-8, influenza A-2 subtype strain Budapest 12/59, influenza A-2 subtype strains Singapore 1/57 and Singapore 4/57, parainfluenza virus type 1 strain Sendai, Newcastle disease virus (NDV) strain "H". All strains were maintained by serial passages in the allantoic cavity of the chick embryo. The harvested allantoic fluids were stored at -70°C until used.

Toxicity assay. (a) In surviving CAM cultures a modification of the method described by TAMM [5] and KÜCHLER and PRACEUS [6] was employed. Fragments of CAM obtained from 12–14 days old chick embryos were suspended and distributed into test tubes in 1 ml volume each. The substance to be examined was added to the medium in various final dilutions. From each dilution 4 parallel cultures were set up. The tubes were incubated for 40 hours at 35–37°C under continuous shaking (Gyrotory shaker, New Brunswick Scientific Co.) at 180 r.p.m. Evaluation was made macroscopically and the toxic effect was designated by one to four crosses, according to TAMM [5].

(b) Monolayer tube cultures of human embryonic fibroblast or permanent amnion cells were covered with 1 ml medium containing various dilutions of the test substance. The cultures were examined daily during a period of 7 days. The medium was not changed during this period. The degree of toxicity was designated by crosses.

Examination of virus inhibitory activity. This was performed by the roller drum infectivity titration method of HORVÁTH [4]. To each ampoule 0.5 ml medium was added together with 1 drop CAM tissue and 0.1 ml virus dilution. Eight parallel ampoules were set up for each virus dilution and for each concentration of the test substance. The virus dilution and the test substance were added to the tissue approximately simultaneously. Results were evaluated on the 3rd day of incubation by testing the haemagglutinating activity with TAKÁTSY's micro-method [7]. Infectivity titres were calculated according to REED and MUENCH [8].

The growth inhibitory activity of the compound was determined as the ratio of infectivity titres in the control and the treated cultures. The limit of error of the method was 0.5 log unit.

Selective inhibitory action assay. The selective effect was expressed as the activity index:

$$\frac{2 + \text{toxic effect inducing concentration in CAM}}{\text{lowest virus inhibitory concentration}}$$

Growth curves. Fragments of CAM suspended in medium [4] were shaken in an Erlenmeyer flask after infection with PR-8 virus. Adsorption was allowed to take place for 1 hour at 35°C. Unadsorbed virus was removed by repeated washing. Incubation was then continued in a fresh medium and samples were withdrawn every other hour. The samples were stored at +4°C overnight and titrated on the next day by the roller drum method. The final concentration of the test substance was 50 µg/ml. The substance was added either simultaneously with, or 1, 2, 4 hours after, the infection.

Results

Toxic effects as related to time and concentration in primary human embryonic fibroblast and permanent human amnion cell cultures are shown in Tables I and II.

Table I

Toxicity of compound H-253 for primary human embryonic fibroblast cell cultures

Concentration, µg/ml	Time of incubation (days)						
	1	2	3	4	5	6	7
100	0	0	+	++	++++	++++	++++
50	0	0	0	+	+++	++++	++++
25	0	0	0	0	+	+++	++++
12.5	0	0	0	0	0	++	++++

The substance was found to be toxic for both types of cell, the effect being, however, dependent on the concentration and the time of incubation.

Table II*Toxicity of compound H-253 for permanent human amnion cell cultures*

Concentration, $\mu\text{g/ml}$	Time of incubation (days)						
	1	2	3	4	5	6	7
100	0	+++	++++	++++	++++	++++	++++
25	0	++	++++	++++	++++	++++	++++
6.25	0	0	0	0	0	0	0
1.56	0	0	0	0	0	0	0

The permanent amnion cell line appeared to be more sensitive than the primary fibroblasts.

In Table III is shown the virus inhibitory effect of the compound on different myxovirus strains. The figures in the Table represent the difference of infectivity titres of control and treated cultures expressed in log units.

Table III*Effect of compound H-253 on the growth of various myxovirus strains*

Concentration, $\mu\text{g/ml}$	Influenza				Parainfluenza type 1 (Sendai)	NDV
	A-0 (PR-8)	Budapest 12/59	Singapore 1/57	Singapore 4/57		
50	1.625	1.750	2.000	3.750	3.500	3.625
25	0.125	1.000	1.250	3.750	2.250	2.375
12.5	0.000	0.125	0.125	2.500	0.625	0.625
6.25	n. e.	n. e.	n. e.	0.750	n. e.	n. e.

n. e. = not examined

Except for strain PR-8, all viruses were considerably inhibited by the compound at 25 $\mu\text{g/ml}$ concentration. Strain A-2 (Singapore 4/57) was the most sensitive, being markedly inhibited at concentrations as low as 12.5 $\mu\text{g/ml}$. It should be noted that strain PR-8 exhibited the lowest sensitivity to the other benzthiazole derivatives, too.

As evaluated according to TAMM, a two plus toxicity for CAM was produced by the substance at 750 $\mu\text{g/ml}$ concentration. Inhibition of viral growth was examined also on CAM, therefore this toxicity limit was considered in the evaluation of the activity index. In Table IV are shown activity indices for various myxovirus strains.

Table IV

Effect of compound H-253 on various myxovirus strains, expressed in terms of activity index

Virus strain	Activity index
Influenza A-0 (PR-8)	15
Influenza A-2 (Budapest 12/59)	30
Influenza A-2 (Singapore 1/57)	30
Influenza A-2 (Singapore 4/57)	120
Parainfluenza-1 (Sendai)	60
NDV	60

The greatest inhibition was found with influenza A-2 (Singapore 4/57) strain, the lowest with influenza A-0 (PR-8) strain.

Direct antiviral activity was tested by incubating for 3 hours at 35°C allantoic fluids containing influenza A-0 (PR-8) or A-2 (Singapore 1/57) strains after addition of the test substance at 50 µg/ml final concentration. The infectivity of the suspensions was then titrated. The titre decrease at the end of the incubation was 0.750 log units for both treated and control suspensions. Haemagglutinating activity remained unchanged throughout.

Action on the activity of viral neuraminidase. The test was performed according to HOFFMANN *et al.* [9]. Results are shown in Fig. 1.

As shown in Fig. 1, no elution occurred at 4°C, while at 37°C all adsorbed virions eluted in 3 hours. The phenomenon was not affected by 50 µg/ml final concentration of the test substance.

It was then examined how the virus inhibitory action was influenced by the addition of the inhibitor at various points of time other than the addition of the virus. These tests were performed in 9-hour growth cycle experiments. Results are shown in Fig. 2.

The inhibitory action of the compound was unaffected if added immediately after the adsorption. Two hours after virus infection the inhibitory effect on strain PR-8 was still considerable. After 4 hours there was hardly any effect demonstrable.

For the sake of completeness, experiments were performed also in mice. Groups of 20 mice each of 20–25 gram weight were infected intranasally with 10 LD₅₀ of strain Singapore 4/57 of influenza A-2 virus. Simultaneously with the infection, treatment of the mice with the compound was started. One group of mice received 1 mg H-253, the other 1 mg of H-253 twice daily, intraperitoneally. The third group serving as control received a daily intraperitoneal injection of saline. Average survival time was uniformly 6 days for all the 3 groups. The virus content of the lungs of the succumbed animals was uniformly 10⁴ ID₅₀, irrespective of treatment with H-253.

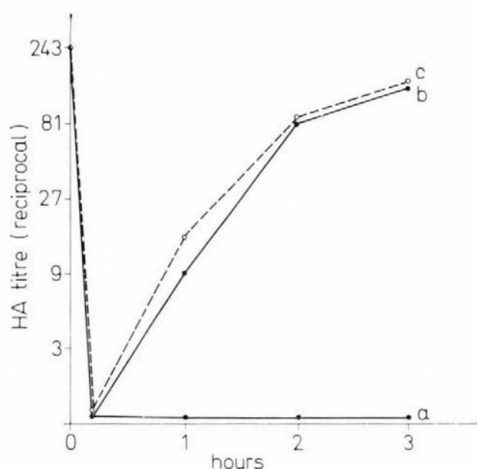


Fig. 1. Elution of A-0 (PR-8) influenza virus strain from chicken erythrocytes in the presence of compound H-253. a) Control at 4°C; b) control at 37°C; c) H-253 50 µg/ml

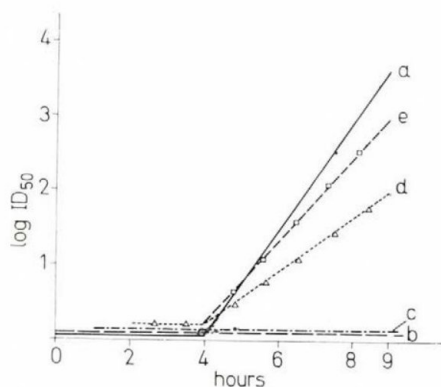


Fig. 2. Effect of compound H-253 on growth cycle of PR-8 influenza virus strain. a) Control; b) the active substance was added simultaneously with viral infection; c) the active substance was added 1 hour after viral infection; d) the active substance was added 2 hours after viral infection; e) the active substance was added 4 hours after viral infection.

Discussion

It appears to be established that the examined benzthiazole derivative inhibited growth of some myxovirus strains *in vitro*. Its effect on influenza A-2 Singapore 4/57 seemed to be considerable. This mouse adapted strain was found most sensitive in other experiments, too.

The substance had no direct virucidal effect. Neither did it affect the neuraminidase activity of strain PR-8. It was an equally active inhibitor if

given prior to, or after, the adsorption. Thus it did not affect the attachment of virions. If added 2 hours after the infection, it was still considerably inhibitory, while after 4 hours it was no more active. This seems to suggest that the substance inhibited some process during the initial intracellular replication phase and was inactive during the maturation phase.

As referred to the toxicity observed in CAM, the substance exhibited a selective virus inhibitory effect *in vitro*. *In vivo*, however, it failed to protect mice. This fact, as well as its toxicity for primary human fibroblast and permanent human amnion cell cultures suggested that the study of this substance is at present chiefly of theoretical interest, particularly because no such effect of benzthiazole derivatives has yet been reported.

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FURTHER STUDIES ON THE LOCALIZATION AND IMMUNOLOGICAL PROPERTIES OF ANTIGENS INDUCED BY HERPES SIMPLEX VIRUS IN HEP-2 CELLS

By

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Summary. Formation of cytoplasmic, perinuclear, diffusely distributed intranuclear and large granular antigens localized along or near the nuclear membrane was observed 3–4½ hours after inoculation with herpes simplex virus in HEP-2 tissue culture cells, while inclusion-like intranuclear antigen appeared at 4½–5 hours after infection, when preparations were stained with sera of guinea pigs immunized with the extract of herpes simplex virus infected tissue culture cells, using indirect immunofluorescent technique. With the same immune serum, perinuclear and finely distributed intranuclear immunofluorescent elements were only found in cells treated with cytosine arabinoside.

With the agar-gel immunodiffusion technique, three antigenic components were separated from the early extract prepared 4½ hours after inoculation, while 6 components from the late extract prepared 16 hours after infection with herpes simplex virus of HEP-2 cells.

Three of the five immune sera used in our tests reacted with all the immunofluorescent elements described above, while one of them did not stain the intranuclear inclusion-like antigen, and the other reacted only with those which are localized in the cytoplasm.

The use of HEP-2 cell cultures to study the multiplication of herpes simplex virus is preferable to that of BS-C-1 cells since in the former large amounts of detectable virus induced antigens are produced.

In a previous paper [1] we have reported on the localization of five immunofluorescent elements in herpes simplex virus (HSV) infected BS-C-1 cells different in morphology and immunological specificity. Our findings partly agreed with those of ROIZMAN *et al.* [2] except that we failed to find large intranuclear granules along the nuclear membrane, and in our preparations the antigens described as large, perinuclear granules were infrequent. In our experiments the intranuclear diffusely distributed granules appeared earlier than the inclusion-like ones. In our study, BS-C-1 cells were used, while the HEP-2 cell line was studied by ROIZMAN *et al.*

According to SABIN's data [3] production of virus induced tissue culture cell antigens is much richer in HEP-2 than in BS-C-1 cells. Based on this finding, we present our further studies on the localization of HSV-induced antigens formed in and without the presence of cytosine arabinoside in HEP-2 cells using the indirect immunofluorescent technique, completing our tests with the use of "Anti-herpes rabbit serum 5685/VIII", applying the agar-gel immunodiffusion technique.

Materials and methods

Tissue cultures. (1) Primary guinea pig embryonic fibroblast cell culture. Two weeks old embryos were treated with 0.2% trypsin solution as described previously [1]. 10^7 cells were added in 1 litre Roux flasks to 80 ml of growth medium (HANKS' balanced salt solution containing 0.5% lactalbumin hydrolysate DIFCO, 20% guinea pig serum, and 0.04% NaHCO_3). The medium was substituted on the second day with the same medium with 0.125% NaHCO_3 content. Confluent dense cell sheets were formed 7–8 days after planting.

(2) BS-C-1 stable line of *Cercopithecus* monkey kidney cell (kindly supplied by Dr. A. B. SABIN) cultures were grown in 1 litre Roux flasks as described previously [4].

(3) HEp-2 cell line (kindly supplied by Dr. I. BÉLÁDI) grown in 1 litre Roux flasks by planting about 10^7 trypsinized cells in 80% of PARKER's medium No. 199 (0.125% NaHCO_3) containing 10% calf serum. The medium was changed 2 and 5 days after planting.

(4) Coverslip cultures of BS-C-1 and HEp-2 cell lines were prepared as described previously [5].

Virus. Syncytium-forming HSV-532 (kindly supplied by Dr. I. BÉLÁDI) in passages 541–543 on HEp-2 cell cultures with an infective titre of $10^{7.5}$ TCD₅₀ per 0.1 ml, when titrated on primary human embryonic fibroblast cells.

Immunization of guinea pigs with the extract of HSV infected tissue culture cells. Guinea pigs were immunized with the 20% extract of guinea pig embryonic fibroblast cell cultures infected with HSV [1]. One ml of antigen was added to an equal volume of complete Freund adjuvant, and injected intramuscularly 3 times at 10 day intervals. Thirty days after the third dose, 1 ml of antigen was injected intramuscularly without adjuvant. Ten days later the animals were bled.

Immune sera. Anti-HSV-infected cell extract immune guinea pig sera designated F-T, and S derived from animals immunized with the extract of HSV infected tissue cultures prepared 5 1/2 hours after inoculation [1]. Guinea pig immune sera designated F and G derived from animals which were immunized with the extract of HSV infected tissue culture cells prepared 15 hours after infection. Serum of guinea pig immunized with the extract of uninfected guinea pig embryonic fibroblast tissue culture cells was designated F-R. Anti-HSV rabbit immune serum designated 5685/VIII kindly supplied by Dr. P. WILDY.*

Inoculation, fixation and staining of tissue culture cells cultivated on coverslips for indirect immunofluorescence technique. Tissue cultures were inoculated with a virus multiplicity of 10 TCD₅₀. After adsorption at 37°C for 30 minutes, the inoculum was poured off, and the cell sheets were washed three times with phosphate buffered saline of pH 7.2 to remove unadsorbed viruses. Then 2.5 ml of BS-C-1 maintenance medium was added into each coverslip tube (Karcag, Glass Factory). The medium added to half the number of the coverslip tubes containing 10 µg per ml of cytosine arabinoside. Coverslips were fixed 4 1/2 and 16 hours after inoculation, and then were examined by indirect immunofluorescence, as described previously [5].

Agar-gel immunodiffusion test. 4 ml of 0.5% melted DIFCO special Noble-Agar in M/15 phosphate buffer solution (pH 6.98) containing 0.85% sodium chloride and 0.1% sodium azide was layered on 50 mm × 50 mm glass slides. After the agar had hardened wells were cut in it with a cork borer. The central well containing 0.15 ml of antigen measured 6 mm in diameter. The six peripheral wells 4 mm in diameter contained the immune sera in 0.05 ml amounts. The diffusion distance was 3 mm. The reaction was carried out at room temperature for 72 hours in a moist chamber.

Results

Compartmentalization of HSV induced antigens in HEp-2 tissue culture cells. For the localization of virus-induced immunofluorescent elements coverslip cell cultures 4 1/2 and 16 hours after exposure to 10 TCD₅₀ virus per cell were fixed, and examined by the indirect immunofluorescence method. We succeeded in localizing antigens in 5 different structures of the nucleus and the cytoplasm of HSV infected cells not treated with cytosine arabinoside (in the follow-

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ing CA—), and in 2 different locations in cells which had been maintained in the presence of 10 μ g per ml of cytosine arabinoside (in the following CA+)

The detected antigens in CA— cells appeared as small nuclear granules diffusely distributed throughout the nucleus, large intranuclear granules localized along or near the nuclear membrane, inclusion-like intranuclear masses, perinuclear and diffuse cytoplasmic fluorescence. In CA+ cells they showed a brilliant fluorescence at the nucleo-cytoplasmic junction, and finely distributed granular fluorescence in the nucleus. Three of our five immune sera reacted with all the immunofluorescent elements formed in virus infected cells. Immune serum S was selected for demonstrating the antigens. The different reactivity of two of the remaining four sera with the immunofluorescent elements allowed the differentiation of these components.

The main characteristics of these antigens according to their indirect immunofluorescence can be summarized as follows. (1) The diffusely distributed granules in the nucleus of CA— cells were easily visible in the preparations fixed 4½ hours after inoculation. As many as 40–45 granules per nucleus were present in about 75% of the cell nuclei. The granules varied in size and were distributed diffusely (Fig. 1a). (2) Large intranuclear granules, mainly localized along the nuclear membrane, were also present in some of the nuclei of the cells seen in Fig. 1a; they were best visualized in preparations stained with immune serum 5685/VIII (Fig. 1b). (3) Brilliant fluorescence at the nucleo-cytoplasmic junction was seen in preparations of CA— cells fixed not later than 4½ hours after inoculation (Fig. 1a). (4) The diffuse cytoplasmic fluorescence was present at both the early and the late phases of the infection in CA— cells (Fig. 1a, b). (5) The inclusion-like, amorphous mass in some of the CA— cells appeared 4½ hours after inoculation. Maximal intensity was observed 6 hours after infection; at 16 hours the inclusion-like bodies looked less dense than before. There was a narrow unstained zone between the antigen and the nuclear membrane. The antigen did not fill the entire centre of the nucleus; there usually were small spots in between, which seemed to be connected to the nuclear membrane by narrow unstained processes (Fig. 1c). (6) Perinuclear rather than diffuse cytoplasmic fluorescence was observed in CA+ cells as early as 3–4½ hours after inoculation (Fig. 1d). (7) Finely granular, diffusely distributed intranuclear fluorescence was present in CA+ cells 4½ hours after inoculation and later. No larger intranuclear granules were seen in these preparations (Fig. 1d).

Immunological reactivity of herpesvirus antigens with different kinds of anti-HSV immune sera. As mentioned previously, we used 5 different immune sera for the localization of immunofluorescent elements in cells infected with HSV. Immune sera S, F-T, and 5685/VIII reacted with all the distinct structures containing the above described antigens. Immune serum F reacted only with the perinuclear and cytoplasmic antigens and failed to stain the intranuclear

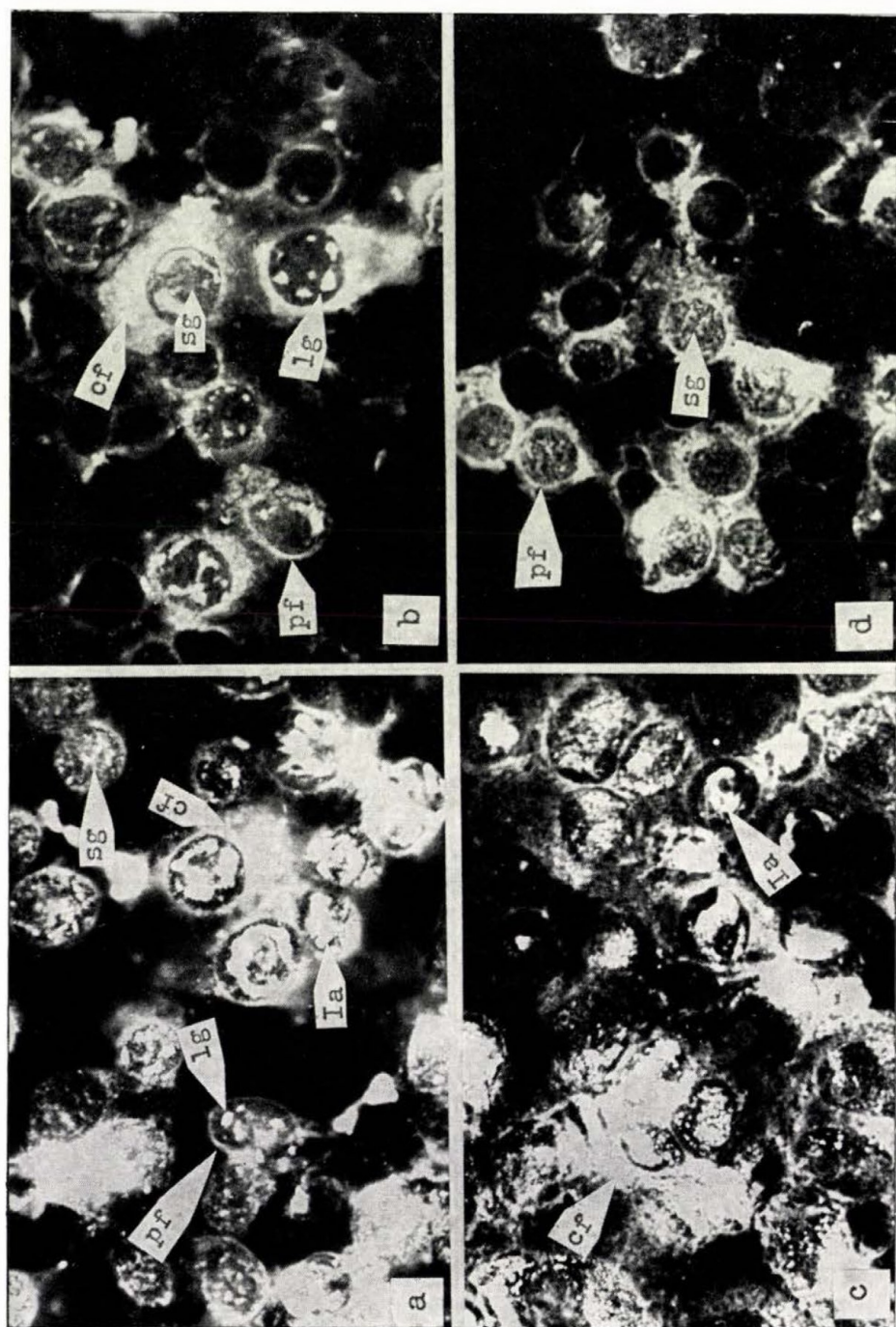


Fig 1.

elements (Fig. 2a). Immune serum *G* reacted with all the antigens present in CA— cells except with the inclusion-like intranuclear one (Fig. 2b). In CA+ cells this serum showed both perinuclear and finely dispersed intranuclear fluorescence (Fig. 2c).

All the tests were made simultaneously and repeatedly. No specific fluorescence was observed in cells stained with guinea pig serum *F-G* inoculated with the extract of uninfected guinea pig embryonic tissue cultures. The immune sera did not show specific fluorescence when uninfected tissue culture cells were stained by them.

The reactivity of the immune sera used in the tests with the above mentioned immunofluorescent elements are summarized in Table I.

Table I

Reactivity of immune sera with immunofluorescent elements induced by herpes simplex virus in HEp-2 cells

Immune sera used	Immunofluorescent element induced in						
	cells treated with cytosine arabinoside		untreated cells				
	Perinuclear	Diffuse granular intranuclear	Diffusely distributed intranuclear	Intra-nuclear large granules	Intra-nuclear inclusion-like	Peri-nuclear	Diffuse cytoplasmic
<i>5685/VIII</i>	+	+	+	+	+	+	+
<i>S</i>	+	+	+	+	+	+	+
<i>F-T</i>	+	+	+	+	+	+	+
<i>F</i>	+	—	—	—	—	+	+
<i>G</i>	+	+	+	+	—	+	+

Comparison of immunofluorescent elements induced by HSV in HEp-2 and BS-C-1 tissue culture cells. With immune serum *S* we made simultaneous tests to localize the immunofluorescent elements in HEp-2 and BS-C-1 tissue culture cells. Results were as follows. In BS-C-1 cells not treated with cytosine arabinoside neither perinuclear fluorescence nor intranuclear large granules localized along the nuclear membrane were observed. The diffusely distributed intranuclear granules consisted of relatively large granules. The inclusion-like

Fig. 1. HEp-2 tissue culture cells fixed at different intervals after inoculation with HSV. Magnification, about 1 : 500. a) CA— tissue culture cells 4½ hours after inoculation, stained with immune serum *S*. b) CA— tissue culture cells 4½ hours after inoculation, stained with immune serum *5685/VIII*. c) CA— tissue culture cells 16 hours after inoculation, stained with immune serum *S*. d) CA+ tissue culture cells 4½ hours after inoculation, stained with immune serum *S*. pf = perinuclear fluorescence; sg = diffusely distributed intranuclear small granules; lg = intranuclear large granules localized along or near the nuclear membrane; cf = cytoplasmic fluorescence, Ia = inclusion-like intranuclear antigen

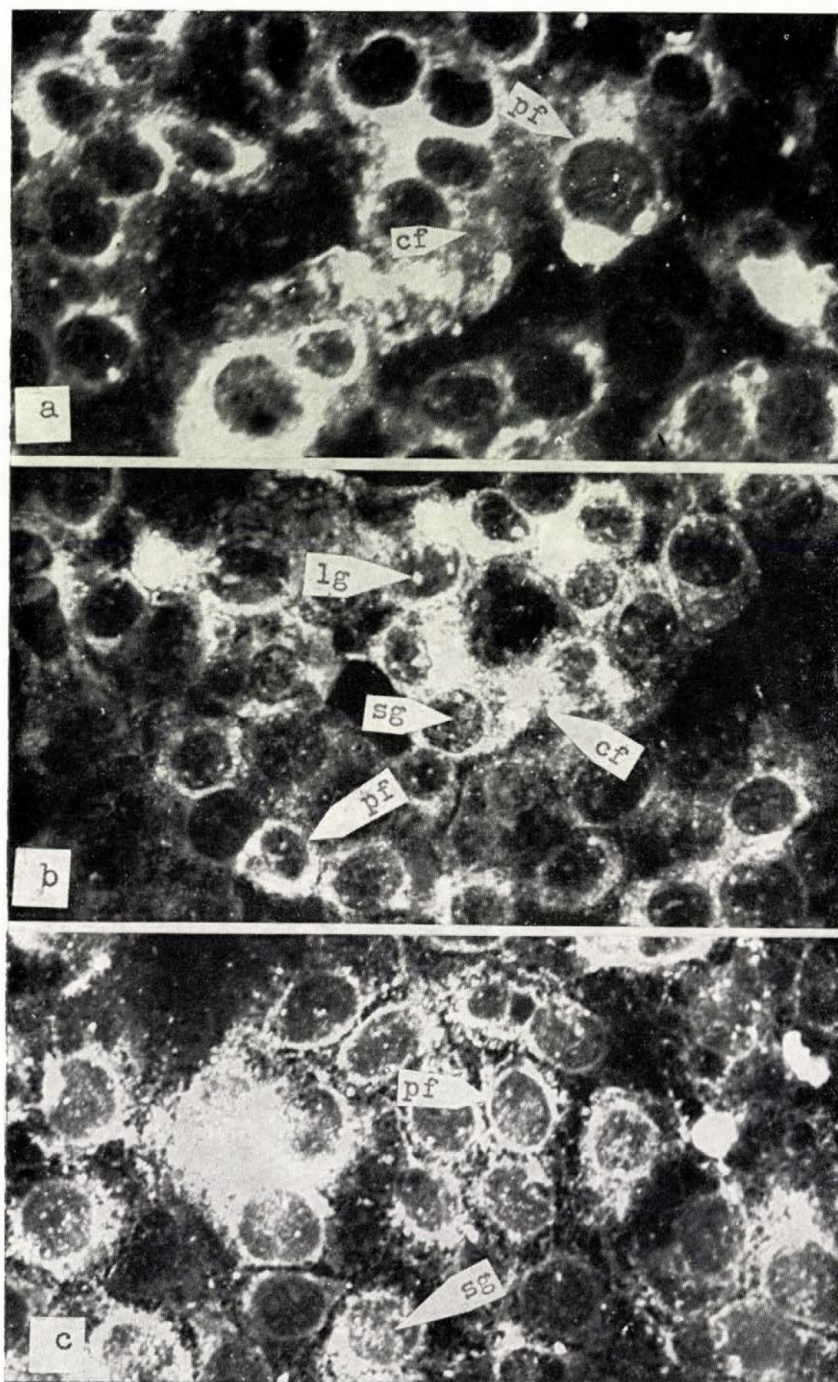


Fig. 2

intranuclear antigens looked less dense in HEp-2 cells than in BS-C-1 tissue cultures. No significant difference was observed in the appearance of the perinuclear and finely distributed intranuclear antigens formed in CA—HEp-2 or BS-C-1 cells. The differences in the appearance of the immunofluorescent antigens formed in HEp-2 and BS-C-1 cells are summarized in Table II.

Table II

Comparison of immunofluorescent elements induced by herpes simplex virus in HEp-2 and BS-C-1 cells when stained with immune serum S

Immunofluorescent elements	Tissue cultures			
	HEp-2		BS-C-1	
	CA—	CA+	CA—	CA+
Intranuclear diffusely distributed granules	+	+	+	+
Intranuclear large granules	+	—	—	—
Intranuclear inclusion-like mass	+	—	+	—
Perinuclear	+	+	—	+
Cytoplasmic	+	—	+	—

CA— = untreated cells

CA+ = cells treated with 10 µg per ml of cytosine arabinoside

Separation of HSV induced cellular antigens by means of agar-gel immunodiffusion. By means of the immunodiffusion technique we succeeded in demonstrating three virus specific antigens when immune serum 5685/VIII reacted with the extract of HSV infected HEp-2 tissue culture cells prepared 4½ hours after inoculation. The tissue culture was maintained in the presence of 10 µg per ml of cytosine arabinoside from the time of inoculation. The same cell extract showed only one precipitation line when reacted with immune sera designated F-T, S, F, and G. When the extract of HSV infected cells prepared 16 hours after inoculation and incubated without cytosine arabinoside was placed into the central well and was allowed to react with immune serum 5685/VIII, precipitin bands were produced by 6 separate antigens differing in rate of diffusion. Immune serum S showed 2, F showed 3 precipitation zones with the extract, while with sera F-T and G only 1 precipitation band was formed (Fig. 3a, b).

Fig. 2. HEp-2 tissue culture cells fixed at different intervals after inoculation with HSV, stained with immune sera F and G. Magnification, about 1 : 500. a) CA— tissue culture cells 4½ hours after inoculation, stained with immune serum F. b) CA— tissue culture cells 16 hours after inoculation, stained with immune serum G. c) CA+ tissue culture cells 4½ hours after inoculation, stained with immune serum G. Abbreviations as in Fig. 1

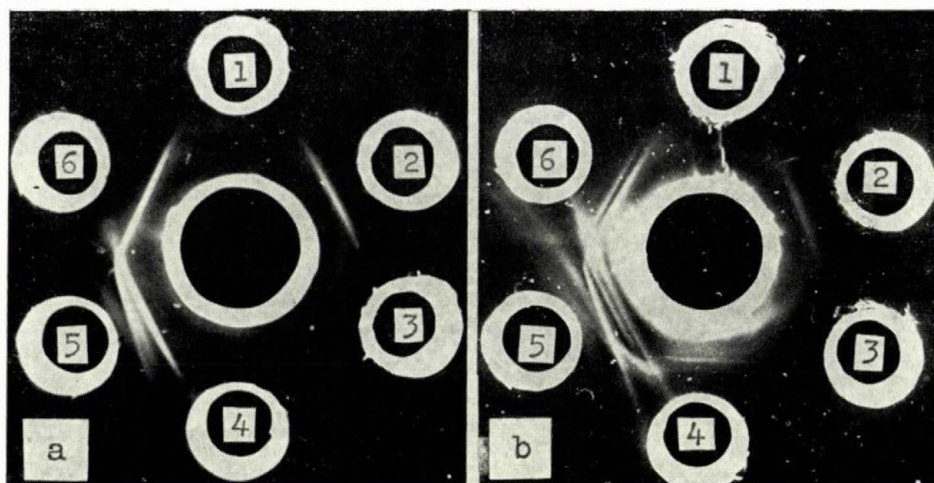


Fig. 3. Reaction of immune sera used for immunofluorescence with the extracts of HSV infected tissue culture cells in the agar-gel immunodiffusion test
Abbreviations: 1 = control serum; 2 = immune serum *F-T*; 3 = immune serum *G*; 4 = immune serum *F*; 5 = immune serum *5685/VIII*; 6 = immune serum *S*. In the central well of Fig. 3a: extract of CA+ cells prepared 4½ hours after inoculation, in that of 3b: extract of CA- cells prepared 16 hours after inoculation

Discussion

We have observed the intranuclear, perinuclear and cytoplasmic localization of 3 immunofluorescent elements in BS-C-1 cells not treated with cytosine arabinoside, and the formation of 2 antigenic components in cells which had been incubated in the presence of the compound [1]. Three hours after inoculation diffusely distributed granules were seen in the nuclei of the CA- cells; the cytoplasm, too, showed diffuse fluorescence. Four hours and more after infection, the nuclei were filled with dense, inclusion-like elements. In CA+ cells only perinuclear and diffusely distributed fine granular fluorescent elements had developed 3 and 4 hours after inoculation, when the preparations were stained with the same immune serum. Adsorption of the serum with the boiled extract of HSV infected tissue culture cells was found to eliminate the reaction with the intranuclear inclusion-like antigenic structure.

Our findings on BS-C-1 cells agreed only partly with those of ROIZMAN *et al.* [2] who used HEP-2 tissue culture cells in their experiments. We observed the appearance of the intranuclear, diffusely distributed granular antigens earlier than that of inclusion-like elements. We failed to find large, intranuclear granules along or near the nuclear membrane of BS-C-1 cells. Antigens described as large, perinuclear granules were rarely seen in our preparations.

According to SABIN [3], BS-C-1 cells are hardly suited for the study of synthesis of herpes simplex antigens since in HEp-2 and primary rabbit kidney cells 4 to 8 times higher concentrations of the different CF antigens were obtained as estimated with their particular serum.

In the present experiments, we succeeded in localizing the same number of immunofluorescent elements in HEp-2 cells as ROIZMAN *et al.* [2] did. There were some discrepancies concerning the time of appearance of some of the antigenic components. In addition, we succeeded to localize two kinds of elements the synthesis of which was not inhibited by cytosine arabinoside. These antigens were separated on the basis of morphology, localization, and immunological reactivity with different kinds of immune serum.

The granules distributed diffusely in the nucleus of CA— cells appeared earlier than the inclusion-like intranuclear mass did. This finding agrees with our previous results obtained with BS-C-1 cells [1] but disagrees with ROIZMAN's findings, who noticed the appearance of the inclusion-like mass 1–2 hours earlier than that of the diffuse granularity [2]. The intranuclear granules seem to be antigenically different from the inclusion-like intranuclear mass, as serum G reacted with these components, while it did not stain the inclusion-like particles. There was some difference in the appearance of the diffusely distributed granules formed in BS-C-1 and HEp-2 tissue culture cells. While those present in BS-C-1 cells were uniformly large, the others formed in HEp-2 cells seem to be the mixture of relatively large granules and finely distributed granular material.

The nuclear amorphous mass may be considered a separate nuclear antigen as immune serum G did not react with it, though it stained the diffusely distributed granules in the nucleus. This means that both kinds of intranuclear antigen were present simultaneously in the nuclei, but the diffuse granular material was overlapped by the dense, diffusely distributed element. The probable identity of this inclusion-like formation with that detected by ROIZMAN *et al.* [2] in HEp-2 cells using anti-boiled cell extract immune serum was shown previously [1].

The large intranuclear granules localized along or near the nuclear membrane seem to be identical with those described by ROIZMAN *et al.* [2]. We did not observe such elements in BS-C-1 cells infected with HSV. Their different nature from the inclusion-like elements was shown by the fact that this antigenic component was stained by immune serum G which did not react with the inclusion-like antigen.

Perinuclear fluorescence was seen in both CA— and CA+ cells soon after inoculation. On the contrary, in BS-C-1 cells fluorescence at the nucleocytoplasmic junction was seen only in cells treated with cytosine arabinoside [1]. Whether this means that the perinuclear elements formed in CA— and CA+ cells were identical but had been produced in higher amount in

HEp-2 than in BS-C-1 cells, or, else, that they were different antigenic components, remained unclear as all the immune sera used reacted with the perinuclear elements formed in both CA+ and CA- cells.

No difference could be found between the cytoplasmic antigen(s) produced early or late after infection, as all the sera reacted with this component both in the early and the late phase.

The diffuse intranuclear, finely distributed fluorescence of CA+ cells looked similar to that present in the form of fine granules in the nuclei of CA- cells. The fact that immune serum *F* did not react with this component though it stained the perinuclear elements in the CA+ cells, indicates that the formation was antigenically different from the perinuclear one.

We have published observations concerning the perinuclear and intranuclear fluorescing elements formed in the presence of cytosine arabinoside [1, 4]. According to LEVITT and BECKER [6] cytosine arabinoside inhibits the formation of *de novo* DNA in HSV infected BS-C-1 cells. As determined by SABIN and TARRO [3] unlike the SV₄₀ picture, the supernatant of extracts of cells infected with herpesvirus contains a considerable amount of non-sedimentable antigens which are definitely virion and some of which are formed early in the course of infection. Also unlike the SV₄₀ situation they found no DNA inhibitors capable of preventing synthesis of virion antigens even though they may prevent synthesis of complete infectious virus. On this basis we may suppose that the antigens localized early in CA+ cells contain antigenic elements synthesized early which are partly non-virion, partly virion in nature. The "tumour-like" antigen induced by SV₄₀ virus in normal tissue culture cells is localized in the nucleus in the form of diffusely distributed granular fluorescence. KITAHARA *et al.* [7] observed in SV₄₀ infected tissue culture cells incubated at room temperature the accumulation of early tumour-like antigen. We detected intranuclear, diffusely distributed fine granular fluorescence in HSV infected BS-C-1 cells incubated at room temperature, where latent infection developed without the appearance of cytopathogenic effect [8]. Further studies are necessary to determine whether this means the accumulation of early viral and/or non-viral antigenic elements.

According to WATSON *et al.* [9], antisera to specific proteins in HSV infected cells produced up to 12 lines in immunodiffusion tests against infected cell extracts.

On the basis of the reactivity in the agar-gel immunodiffusion test of immune serum 5685/VIII with the extract of CA+ and CA- HSV infected cells there seemed to be at least 3 different antigenic components in the CA+ cells, while 6 were detected in the extract of CA- cells. The remaining 4 immune sera showed less precipitation zones than serum 5685/VIII, though the reaction of two of them with the immunofluorescent elements in HSV infected cells did not differ in any respect. This means that using the agar-gel immunodiffu-

sion test for determining the antigenic components, more potent immune sera are needed than with the use of the immunofluorescent technique.

As mentioned previously, these findings concerning the localization in the cytoplasm and the nucleus of the infected cells of immunologically different antigens at different periods after infection are in accordance with the recent findings of ROIZMAN and KAPLAN, according to whom HSV proteins are most probably synthesized in the cytoplasm and diffuse, or are transported into, the nucleus. When the proteins from the nuclear membrane are transferred into the cytoplasm, they undergo an immunological change. This change may be due to the aggregation of proteins to form the nucleocapsid [10].

The different scale of reactivity of immune sera with the various antigens formed during the infection makes it clear that selection and dilution of the serum used for detecting antigenic components have a great importance, in accordance with the data of SABIN [3], ROIZMAN *et al.* [2] and LEBRUN [11].

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PLAQUE FORMATION BY DIFFERENT MEASLES VIRUS STRAINS IN A MONKEY KIDNEY CELL LINE

By

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Summary. Plaques formed by different strains of measles virus in monolayers of three different cell cultures (primary monkey kidney, HeLa and RUZICKSKA's monkey kidney cell line III/1) were investigated. The plaques formed in the cultures of cell line III/1 were the most characteristic in appearance.

Four strains of measles virus were titrated in parallel in III/1 and HeLa cell cultures by both the tube dilution and the plaque technique, and the neutralizing antibodies of human sera were titrated in parallel in tube and plaque cultures. The results were consistent.

The morphological characteristics of the plaques formed by 5 different strains of measles virus in III/1 cell monolayer were compared. Each strain formed both small and large plaques and the ratio of small plaques to large ones appeared to be characteristic of the strain. Predominance of small plaques does not indicate an *a priori* low virulence for man. From two strains small-plaque and large-plaque clones were isolated.

Since ENDERS and PEEBLES [1] isolated measles virus in human and simian tissue cultures, attempts have been made to propagate the virus in various primary and continuous cell cultures. The plaque technique was first applied by HSIUNG *et al.* [2], using primary monkey kidney cell cultures. UNDERWOOD [3] employed HeLa cells and an overlay composed of chick embryo extract and chicken plasma, whereas DEMAEYER [4] used primary and continuous human amnion cultures and agar overlay. Comparative studies of different measles virus strains have been published by BUYNACK *et al.* [5], RAPP [6], DEMAEYER and ENDERS [7] and FÜRÉSZ and MAREAU [8]. MARES *et al.* [9] investigated the plaque formation in various human diploid cell strains. It has been shown that the measles virus forms well-pronounced plaques in some cell cultures but not in others.

In the present work monolayers of different cultured cells were inoculated with five strains of measles virus. The most characteristic plaques were developed in cultures of RUZICKSKA's monkey kidney cell line No. III/1. Using this cell line, a simple and economical plaque technique was evolved, and applied in investigating the plaque-forming properties of different strains of measles virus.

Materials and methods

Virus strains. 1. The strain Leningrad 4A originated from Professor A. A. SMORODINTSEV (Leningrad). Since 1961 it has been carried over numerous passages in this Institute, first in cultures of the Am 57 cell line [10], subsequently in HeLa cell cultures. 2. The strain E-Bg — as

designated by us — is a descendant of the Edmonston strain. It was maintained in the Virus Department of the Institute of Hygiene, Belgrade, and subsequently adapted to HeLa cell cultures in this Institute. 3. The Philadelphia (Ph) strain was received from the Division of Immunological Products Control, Medical Research Council Laboratory, London. In this laboratory the strain has been maintained on the cell line III/1. 4. The Schwarz strain originated from the commercial Glaxo vaccine and has been maintained in the cell line III/1 by us. 5. The OKI-II strain was isolated by us from leukocytes of a child with measles, and carried over 3 passages in primary monkey kidney cell cultures and subsequently maintained in passages in III/1 cultures.

Cell cultures. Primary monkey kidney cell cultures were prepared according to YOUNGNER's [14] method. The growth medium contained 0.5% lactalbumin hydrolysate and 2% bovine serum in HANKS' balanced solution. HeLa cell cultures were grown in PARKER's medium No. 199 containing 10% bovine serum. The cell line III/1 was developed from rhesus monkey kidney cells [11, 12, 13] and had undergone more than 100 passages until it has acquired stable properties. The medium contained 5% of a 5% lactalbumin hydrolysate solution in a mixture of 10% bovine serum, 45% HANKS' balanced solution and 40% PARKER's No. 199. Primary monkey kidney cell cultures were started with 2×10^5 cells, HeLa and III/1 cultures with 1×10^5 cells per ml per tube. As maintenance medium, PARKER's No. 199 with 0.2% human albumin was used throughout. All cultures were incubated at 37°C.

Monolayers for plaque titration were prepared in glass vials 2 cm in diameter, tightly closed with rubber stopper. The initial cell count was 1.5×10^5 cells in 1.5 ml of the medium given above for the corresponding tube cultures. Incubation temperature was 37°C throughout. Continuous cell layer developed within 1 or 2 days. The medium was then sucked off and the inoculum was plated on the cell layer in a volume of 0.1 ml. After an adsorption period of 2 hours the inoculum was washed off and replaced by a gel, containing 1.2% agar and 0.2% human albumin in PARKER's No. 199 devoid of phenol red. After 4–6 days' incubation, *i. e.*, when characteristic plaques had developed, the cell layer was fixed by adding 10% formalin and the agar overlay was removed by a thin metal spatula. The fixed cell layer was washed, and stained with methyl violet.

Virus titration in tube cultures. Tenfold dilution series were prepared and ten parallel tubes were inoculated with each dilution, 0.1 ml per tube. CPD_{50} was calculated according to the REED—MUENCH formula from the results recorded on the 6th day.

Neutralization tests in tube cultures. Fourfold dilution series were prepared from human sera and each dilution was mixed with an equal volume of virus to contain 100 CPD_{50} per 0.1 ml mixture. The mixtures were kept at +4°C overnight and then three tube cultures were inoculated with each mixture, 0.1 ml per tube. The final reading was done on the 7th day. The highest serum dilution protecting all the three cultures was accepted as titre.

Virus titration by the plaque technique. Five monolayers were inoculated with each member of a tenfold dilution series, 0.1 ml per vial. Final results were read on the 6th day of incubation. PFU value was calculated for 0.1 ml.

Neutralization tests in plaque cultures. Five monolayers were inoculated with each of the virus-serum mixtures prepared for titration in tubes. Results were read on the 7th day. The highest serum dilution in the presence of which no plaque had developed was accepted as titre.

Selection of plaque-variants. To separate different plaque-variants, serial plaque-plaque passages were performed. Various dilutions of virus were inoculated, each into a great number of cultures. Plaques were picked up only from cultures where one or two plaques had grown. Such plaques, visible through the agar sheet without staining, were signed on the bottom of the vial. Then several cu. mm of the agar gel covering the plaque were sucked into a thin-pointed pipette and then suspended in 0.5 ml medium. The suspension was frozen and thawed three times before passaging.

Results

Comparison of plaques developed by measles virus in different cell cultures. The plaques in primary monkey kidney cell cultures could not be observed exactly with the naked eye (Fig. 1a), but the cytopathic effect was well recognizable microscopically (Figs 1b and c). The plaques in HeLa cell monolayers were well visible, but — as shown by microscopic control — their

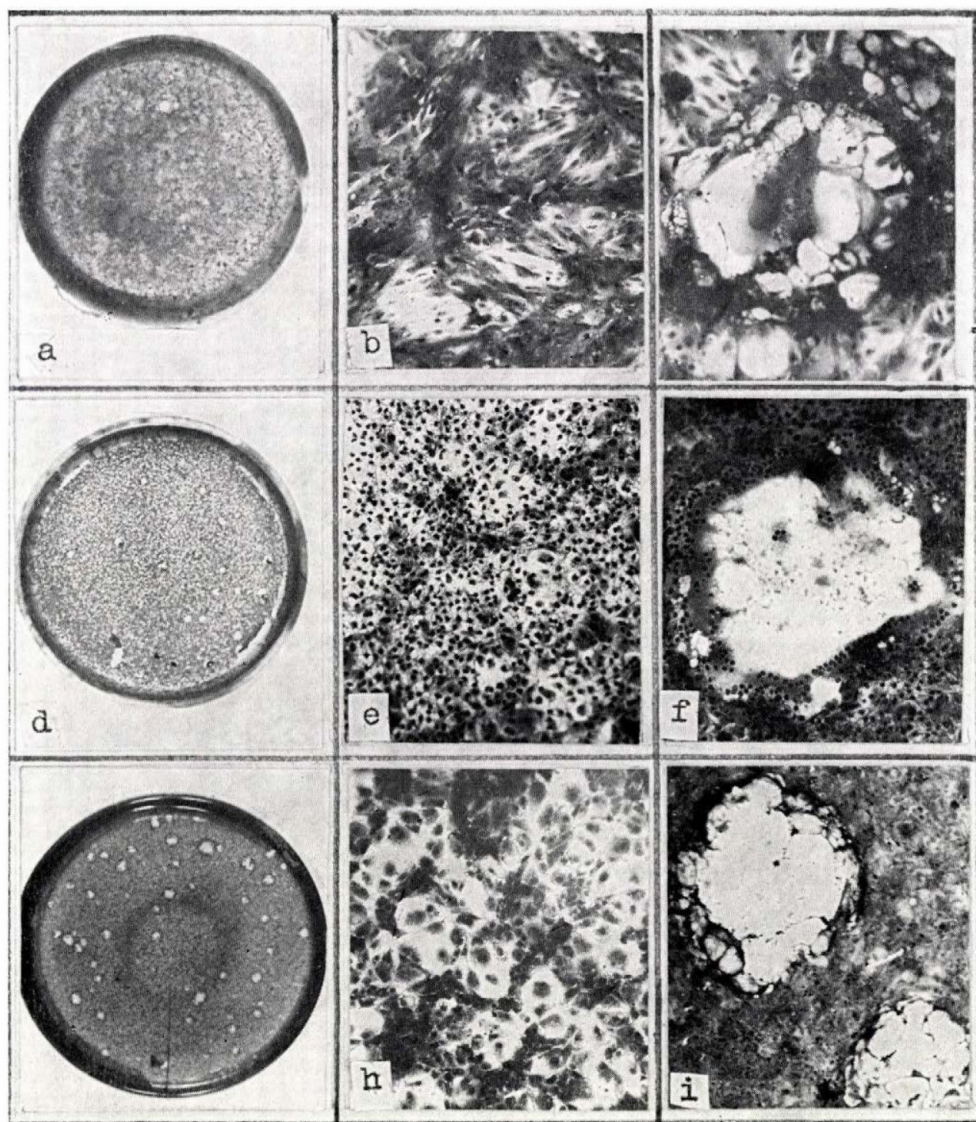


Fig. 1. Plaque formed by E-Bg strain of measles virus in three different cell cultures. a, b and c: Primary monkey kidney culture; d, e and f: HeLa cell culture; g, h and i: cell line III/1. 1st column: Plates $\times 2$, 2nd column: uninoculated cultures $\times 160$, 3rd column: plaques $\times 160$

differentiation from gaps in the monolayer was not quite easy, for some of the syncytia detach and the edge of the remaining tissue is not characteristic enough (Figs 1e and f). The most characteristic, best circumscribed plaques were obtained in the III/1 cell cultures (Fig. 1g). The syncytium in the centre of the plaque soon burst and the nuclei, packed at the periphery of the

plaque, formed a ring which tended to detach separately (Figs 1h and i). Similar phenomena had been observed in another monkey kidney cell line by RAPP [6], who distinguished clear, red and red-bordered plaques. He suggested that the phenomenon is not genotypical. In our opinion the different plaque forms in the present material represent different developmental stages. Photographs and micrographs are shown in Fig. 2. The plaque with stained centre (corresponding to RAPP's red plaque) still contains a syncytium with stained cell debris (Figs 2a and b). In the clear plaque (Figs 2c and d) the syncytium

Table I

Parallel titration of four measles virus strains in tube and plaque cultures of cell line No. III/1

Virus strain	Infectivity titre in	
	log TCD ₅₀ /0.1 ml	log PFU/0.1 ml
Leningrad 4A	4.5	4.7
E-Bg	5.5	5.1
Philadelphia	5.6	5.2
OKI-II	3.5	3.5

has already bursted, whereas the plaques bordered by a dark-stained ring (red-bordered plaques of RAPP) represent the last stage of plaque formation (Figs 2e and f).

Comparative infectivity titrations. Comparative studies were performed to establish whether monolayers in small vials of the cell line III/1 cover the requirement for infectivity titration. Table I shows that the results obtained by different methods in different cell cultures agreed satisfactorily.

Comparative neutralization tests in tube and plaque cultures. Sera of children vaccinated with live measles vaccine were titrated in parallel in tube-cultured HeLa and III/1 cells and by the plaque technique on the III/1 cell line. Table II shows that the results were consistent.

Plaque morphology of different measles virus strains. Since the III/1 line produced the most pronounced plaques, in further studies only cultures prepared from this cell line were used.

Fig. 2g shows that the strains L 4A and E-Bg formed both small and large plaques; the difference between small and large plaques was more pronounced in the case of the latter strain. In the cultures inoculated with the Ph strain, large plaques occurred less frequently. The attenuated Schwarz

Fig. 2. Plaques in III/1 monolayers. a, b: Plaque with stained centre; c, d: plaque with clear centre; e, f: plaque bordered by dark-stained ring. 1st row: magnification $\times 2$; 2nd row: magnification $\times 160$. g: Plaque formed in III/1 monolayers by different strains of measles virus. 3rd row: from left to right L 4A, E-Bg, Philadelphia. 4th row: Schwarz, OKI-II

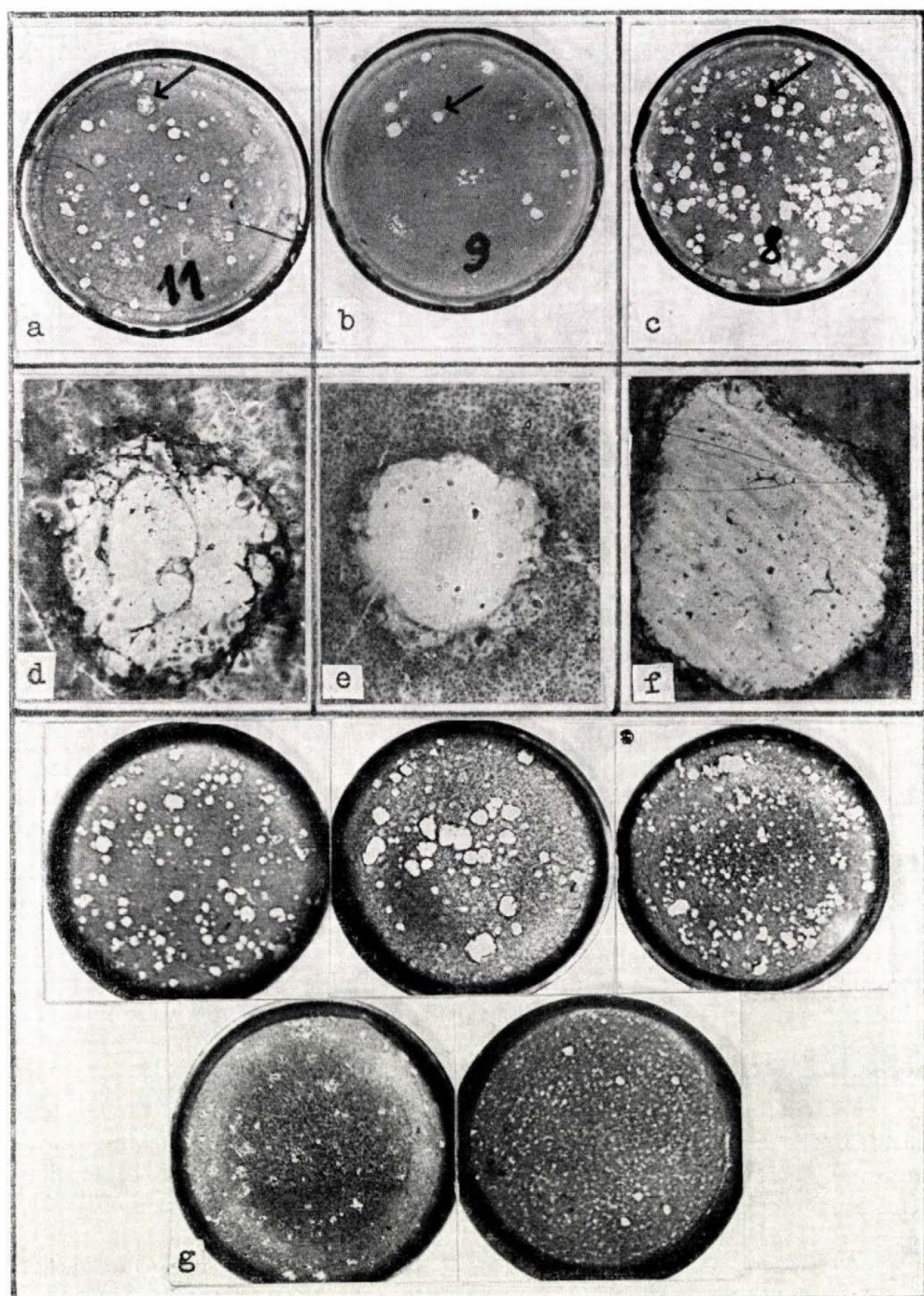


Fig. 2

strain, and the OKI-II strain in its initial passages, formed mainly extremely small plaques and occasionally one or two larger plaques (Figs 3a and c). The large plaques formed by the OKI-II strain were different from those formed by the Schwarz strain (Figs 3b and d) in displaying, in addition to the ring consisting of packed nuclei, an outer ring of foamy appearance. After several passages in III/1 cultures both the small and the large plaques of the OKI-II strain appeared larger (Fig. 3e) than the corresponding plaques of the earlier passages.

Table II

Parallel titration of human sera in tube and plaque cultures

Serum No.	Serum titres obtained in		
	HeLa tube cultures	III/1 tube cultures	III/1 plaque cultures
1	neg.	neg.	neg.
2	neg.	neg.	neg.
3	neg.	neg.	neg.
4	neg.	neg.	neg.
5	1 : 128	.	1 : 128
20	1 : 32	.	1 : 32
42	1 : 128	.	1 : 128
42/A	1 : 512	.	1 : 512
90	1 : 512	.	1 : 512
104	1 : 128	1 : 512	1 : 512
479	1 : 512	1 : 512	1 : 512

. = not tested

It was assumed that the strains under study were composed of at least two plaque variants, and the ratio of the small plaques to the large plaques showed strain differences. To elucidate this question, attempts were made at separating the two variants. However, the small size of the plaques made it difficult to separate the small-plaque variants. In spite of this, we succeeded in separating both variants from two strains, *viz.*, the E-Bg and the OKI-II strains. The large-plaque variant separated from the OKI-II strain formed unusually large plaques. The plaque formation by this strain before separation and by the selected small- and large-plaque variants are shown in Figs 3c, f and g, respectively.

The large-plaque variant of the E-Bg had lost its haemagglutinating capacity while the small-plaque variant had retained it. To check the stability of the separated lines, further plaque passages are in progress.

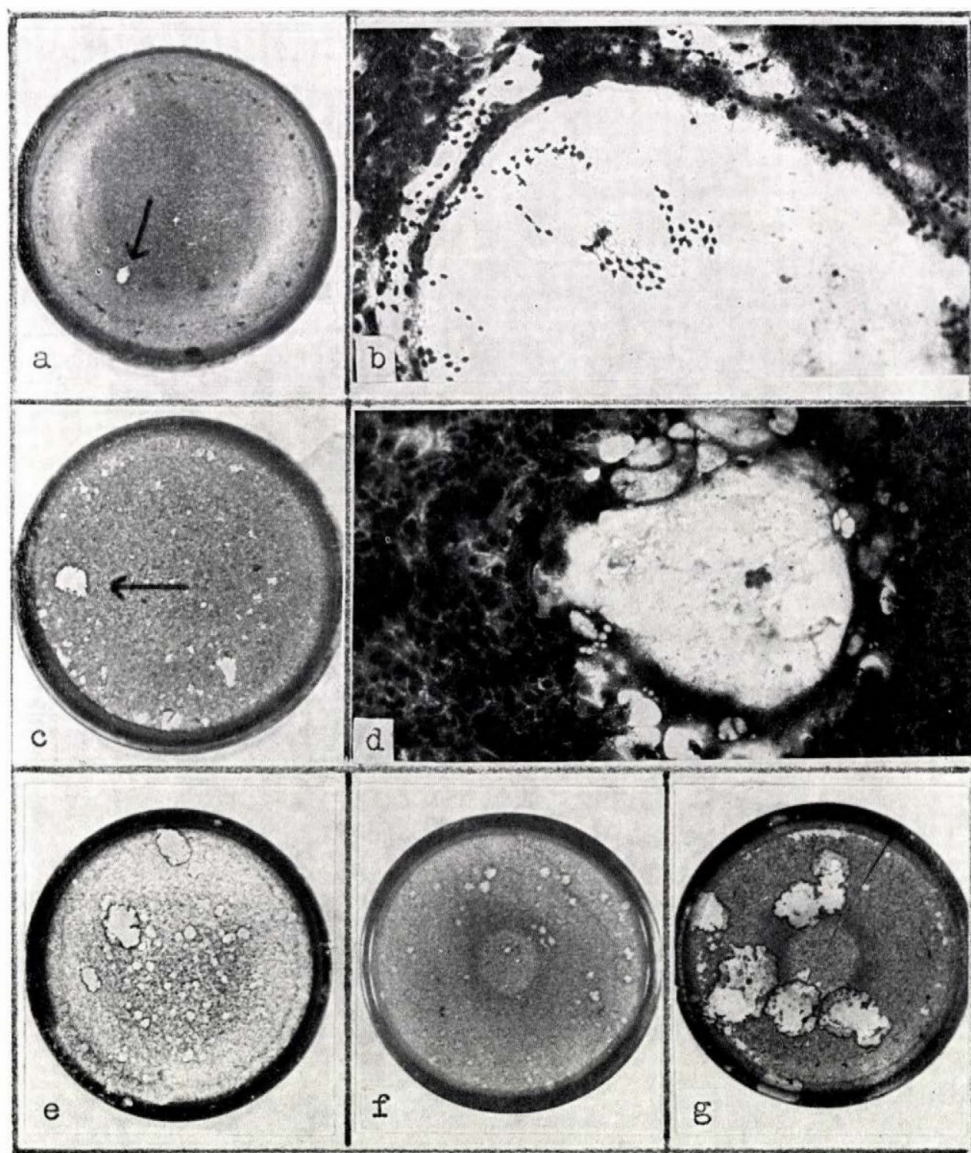


Fig. 3. Plaque morphology in III/1 monolayers. a: Plate inoculated with the attenuated Schwarz strain. The arrow points to the single large plaque $\times 2$. b: The large plaque magnified from Fig. 3a, $\times 160$. c: Plate inoculated with the virulent OKI-II strain. The arrow points to the single large plaque. $\times 160$. d: The large plaque, magnified from Fig. 3c. e: Plaques of varying size before separation, f: small-plaque variant, g: large-plaque variant

Discussion

The monkey kidney cell line No. III/1 proved to be suitable for the investigation of plaque formation by measles virus strains. The use of tightly stoppered small vials represents an economical method, saving room and material and requiring no CO₂ box.

It has been suggested [5, 15] that small-plaque-forming measles virus strains are attenuated, whereas large-plaque-forming ones are virulent, for man. In the present study the highly attenuated Schwarz strain formed mainly very small plaques, and the virulent strains L 4A and E-Bg, both large and small ones. However, on the plates inoculated with the non-attenuated Ph strain formation of small plaques was predominant. Furthermore, like the attenuated Schwarz strain, the freshly isolated wild strain OKI-II produced, in its early passages, almost exclusively small plaques. This latter strain after having been subjected to 20 passages in monkey cell cultures produced large plaques more frequently and both the large and the small plaques showed a well-defined tendency to grow in size, presumably as a result of adaptation to monkey cells.

In this respect it should be noted that the Schwarz strain, the other strain forming the smallest plaques, had adapted itself to fowl cells, *i.e.*, to the cells of a species standing far from the monkey. This strain had not been passaged in III/1 cell cultures before tested for plaque formation.

We suppose that the history of a strain may be responsible for its actual plaque-forming properties in a given cell culture. As regards the possible correlation between formation of small plaques and low virulence for man we agree with DEMAEYER [4] in that only systematic plaque studies of measles virus strains isolated from a number of cases could settle this question in a more definite way.

The plates inoculated with the attenuated Schwarz strain cannot be distinguished from those inoculated with our wild strain if only the size of the large and of the small plaques and the relative frequency of the plaques of different sizes are considered. Nevertheless, there is a pronounced difference in plaque morphology between the two strains: the outer ring of foamy appearance occurs only around the plaques formed by the latter.

We found no direct correlation between the initial rate of growth of plaques or the attainable virus titre and the eventual plaque size, *e.g.*, the small plaques of the Ph strain become visible as early as on the 2nd day, but stop growing soon thereafter. The same strain attains higher infectivity titre than any of the other strains under study, it mainly forms small plaques nevertheless.

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LYTIC FACTOR AND COMPETENCE IN BACILLUS SUBTILIS

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Summary. Well-functioning cells of *B. subtilis* autolysed rapidly after they had been transferred into a poor medium. Highly transformable strains showed a high autolytic activity. The lytic factor changed in activity during cultivation.

Varying with the strain used, there was a 45 to 90 minute difference between the peak of competence and the maximum activity of the lytic factor; the development of competence was more rapid than the rate of lysis.

Extracts of competent cells, filtrates of competent cultures and lysozyme did not enhance the development of competence. Neither primary nor secondary evidence has been found as to whether the lytic factor promoted the development of competence.

Under special culturing conditions *B. subtilis* cells become competent and take up transforming deoxyribonucleic acid from the donor cells. In the course of the development of competence an alteration may occur in the cell wall. The degree of morphological and chemical changes depends on the transformability of the culture [1]. YOUNG and SPIZIZEN [2, 3] demonstrated the presence of a lytic factor in the wall of competent *B. subtilis* cells.

Similarly, an active autolytic enzyme has been found in well-transformable pneumococci [4].

The lytic factor seems to promote the development of competence. The purpose of the present work was to examine the relationship between the lytic factor and the development of competence.

Materials and methods

Strains. *B. subtilis* strains Marburg, 168 M try⁻, SB 25 try⁻ hist₂⁻ and try⁻ [5]. were kindly supplied by Dr. B. S. STRAUSS and Dr. E. W. NESTER. The try⁻ spontaneous mutant of strain 168 try⁻ was isolated in our laboratory.

Culture media. The strains were maintained on potato agar [6]. Precultivation was performed on MGY agar slants. MGY liquid medium was used for the development of competence. Strain XB 25 was grown in MGY medium containing 10 µg l-tryptophan and 10 µg l-histidine per ml. The remaining strains were cultured in MGY medium containing 5 µg tryptophan per ml. T medium was employed for transformation experiments. The transformants were selected on MG agar. Composition of the media has been described in [7].

Cell lysis was assayed in a pH 8 buffer-salt (BS) solution: KH₂PO₄, 0.045%; Na₂HPO₄ · 2H₂O, 1.128%; NaCl, 0.9%.

Preparation of DNA. DNA was obtained from *B. subtilis* Marburg by the phenol extraction method of SAITO and MIURA [8].

Transformation was performed as described by HORVÁTH [7].

Assay of lysis. The organisms were cultured in MGY medium shaken at 100 r.p.m. in a 37°C water bath. Samples were taken at various intervals and centrifuged. The cells were resuspended in BS solution to an optical density (O.D.) of 0.4, then shaken in water bath at 37°C. The rate of lysis was estimated by measuring the change in O.D. The time in hours needed for a 50% reduction of the initial O.D. was regarded as the activity of the lytic factor. The graphs show the reciprocal values of the 50% lysis time in log units. An easy comparison of graphs obtained in this manner with the competency curves allowed an adequate estimation of the relationship between lytic factor and competence.

Cell extraction. Competent and incompetent cells were centrifuged at 1600 g for 6 minutes. The cells were extracted with the same volume of distilled water by grinding with glass powder. The extract was centrifuged at 1600 g for 6 minutes, then the supernatant was removed and used in the experiments.

Results

In examining the relationship between the activity of the lytic factor and competence the following problems have been analysed. (a) Change in the activity of the lytic factor during incubation and the optimum pH of lytic

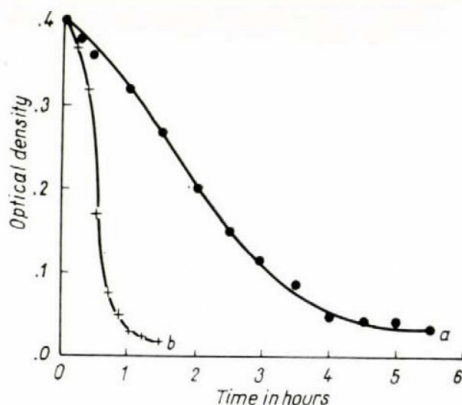


Fig. 1. Lysis of *B. subtilis* SB 25 cells in BS solution after 1 hour 30 minutes (a) and 3 hours (b) incubation in MGY medium

activity. (b) Lysis of cells in T medium containing various amounts of casein hydrolysate. (c) The activity of the lytic factor in various *B. subtilis* strains. Finally, the effect on the development of competence of (d) lysozyme, (e) cell extract, (f) filtrate of competent cultures and whole competent cells has been studied.

(a) *B. subtilis* SB 25 grown on an agar slant overnight was seeded into MGY medium and cultured as described above. After 1½ and 3 hours incubation small samples of the culture were centrifuged at 1600 g for 6 minutes. The deposit was resuspended in BS solution, incubated and assayed for cell lysis (Fig. 1).

Fig. 1 indicates that the time needed for 50% lysis varied according to the duration of incubation in MGY medium. Incubation of competent cells

in BS solution adjusted to different pH values indicated that the lytic activity was most definite in the range of pH 8.

(b) As transformation experiments are generally carried out in media containing low concentrations of casein hydrolysate, it seemed desirable to examine the influence of this substance on cell lysis. *B. subtilis* 168 was suspended in T medium containing 0.1, 0.025, 0.006 and 0% casein hydrolysate (pH 7.1). The cells became lysed in all media (Fig. 2).

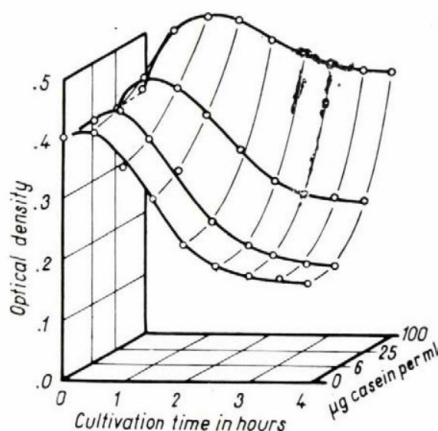


Fig. 2. Lysis of *B. subtilis* 168 cells in T medium supplemented with various amounts of casein hydrolysate

(c) Strains 168, SB 24 and 168 M were inoculated into MGY medium. Samples taken at intervals during cultivation were centrifuged, resuspended and incubated in BS solution as described above. The level of competence was determined in transformation experiments. Results are shown in Figs 3, 4 and 5.

In *B. subtilis* 168, maximum lytic activity occurred at the 3rd hour of incubation; peak competence was observed after 3 hours and 45 minutes. Accordingly, there was a 45 minute difference between the two maximum values. The steepness of the curve representing the rising phase of competence differed from the steepness of the curve expressing cell lysis. The difference between the maximum and minimum values for competence was higher than the difference between the corresponding values for lysis.

The results for strains SB 25 and 168 M were almost identical with those obtained for strain 168, except that the difference between peak competence and lysis was 1 hour 30 minutes for both organisms.

The spontaneous mutant of *B. subtilis* 168 produced larger and rougher colonies than the parent strain; the mutation occurred at a very low frequency. The two cultures differed considerably in growth curve. Chain formation by

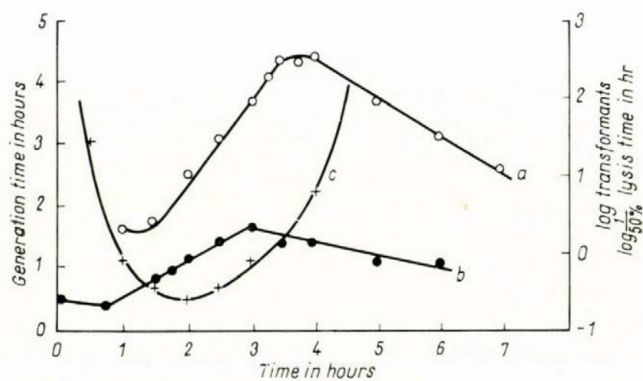


Fig. 3. Change of competence (a), lytic factor (b) and generation time (c) in *B. subtilis* 168

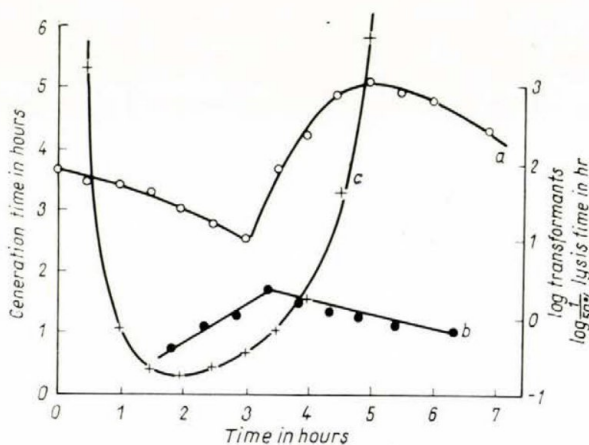


Fig. 4. Change of competence (a), lytic factor (b) and generation time (c) in *B. subtilis* SB 25

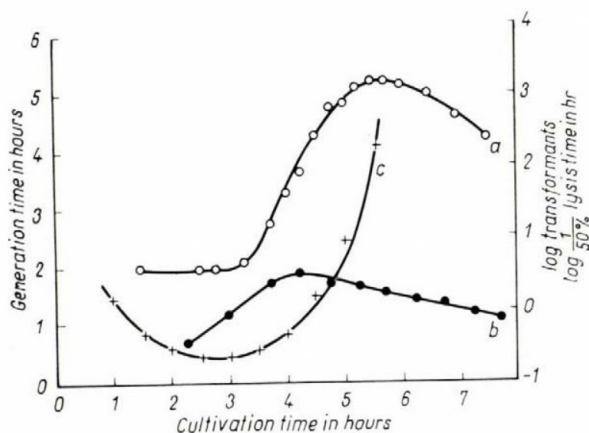


Fig. 5. Change of competence (a), lytic factor (b) and generation time (c) in *B. subtilis* 168 M

the spontaneous mutant was more definite than by the parent strain. The transformation frequency for the spontaneous mutant was tenfold lower than for the parent strain. The time needed for 50% lysis was 2 hours 30 minutes (the corresponding times for strains SB 25 and 168 were 28 and 33 minutes, respectively).

(d) Lysozyme causes lysis by decomposing muramic and teichoic acid present in the cell wall. *B. subtilis* 168 cultured on agar slant overnight was suspended in BS solution containing varying concentrations of lysozyme. Results obtained at various stages of incubation are presented in Fig. 6.

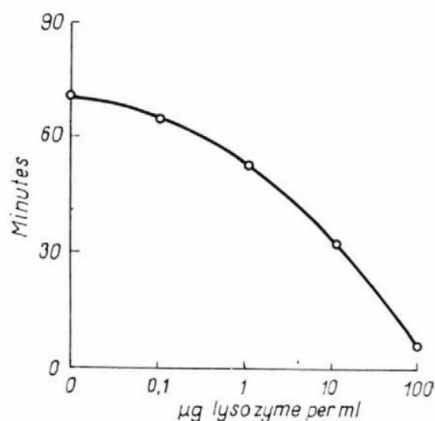


Fig. 6. Lysis of *B. subtilis* 168 cells in BS solution containing various amounts of lysozyme

Figure 6 indicates that the higher the lysozyme concentration the shorter the incubation time needed for 25% lysis.

Transformation was performed in solutions containing different concentrations of lysozyme. At high lysozyme concentrations (16 to 48 µg/ml) the number of *B. subtilis* 168 transformants decreased in a short time. Similarly, no increase was observed in the number of transformants with the spontaneous mutant of strain 168.

(e) Competent cells of *B. subtilis* 168 and SB 25 were centrifuged and extracted. Various amounts of the extracts were added to series of MGY cultures seeded partly with competent, partly with incompetent cells of strains SB 25, 168 and spontaneous mutant 168. In the number of transformants produced there was no significant difference between media with and without extract.

(f) An attempt was made to demonstrate changes in competence during growth in filtrates of competent and incompetent cell cultures.

Liquid MGY medium was inoculated with 1.5×10^7 *B. subtilis* 168 cells per ml. The culture was incubated until the cells had become competent (O.D. = 1.4, 4 hours). Then balanced growth was initiated for 1 hour; finally

the culture was centrifuged at 1600 *g* for 6 minutes and the supernatant was passed through G5 filter. The competent cells were designated as B1, the supernatant as M1.

Another flask containing MGY medium was seeded with 3.3×10^6 *B. subtilis* 168 cells per ml. Cultivation lasted until O.D. = 0.05 had been reached, then the cells were centrifuged and the supernatant filtered as described above. The cells were designated as B2, the supernatant as M2. The filtrates

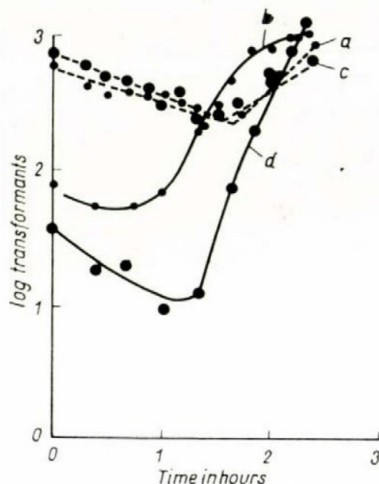


Fig. 7. Development of competence in *B. subtilis* 168 cells precultivated under various conditions. a = M1 + B1; b = M1 + B2; c = M2 + B1; d = M2 + B2

and bacterial suspensions were measured into 4 flasks in the combinations: 1. M1 + B1; 2. M1 + B2; 3. M2 + B1; 4. M2 + B2.

O.D. was adjusted in all flasks to 0.05 and the cells were grown for 3 hours. Samples taken during cultivation were assayed for competence by transformation to prototrophy. After 3 hours incubation O.D. was 0.43, 0.55, 1.6 and 1.6 in the above order of cultures. Results are shown in Fig. 7.

Figure 7 indicates that competent cells yielded essentially the same competency curves. Competence decreased slowly for 2 hours then increased.

In culture M1, which contained incompetent cells, competence began to rise after 1 hour. In medium M2 the rise occurred after $1\frac{1}{2}$ hours. The difference between the two curves can be attributed to a difference in the media rather than to some "factor".

An attempt was made to convert incompetent cells of strain 168 and its spontaneous mutant to competency by the use of whole competent cells of strains 168 and SB 25. Neither primary nor secondary evidence has been found that competence can be transmitted from intact competent cells to incompetent bacteria.

Discussion

YOUNG, SPIZIZEN and CRAWFORD demonstrated in the cell wall of *B. subtilis* the presence of a lytic enzyme lysing the cell wall under certain conditions. During autolysis the amount of NH_2 -terminal l-alanine increased. The autolytic enzyme corresponded to acetylmuramyl-l-alanine amidase. The enzyme exerted lower activity in poorly transformable strains [1, 3, 9].

YOUNG, TIPPER and STROMINGER [9] assumed that the autolytic enzyme caused a localized hydrolysis in the cell wall and thus the negatively charged DNA segment was able to enter the competent cell.

The present data indicate that the lytic factor changed in activity during cultivation. Autolysis occurred rapidly after the cells had been transferred to a poor environment. Well-functioning cells seemed to have been involved in an "accident", that is they autolysed when exposed to non-physiological conditions.

A decrease of the cell-wall precursor in the medium also initiates lysis [10].

Well-transformable strains differed in the appearance of the peak of competence and the highest activity of the lytic factor. The curve representing the rise of competence was steeper than the curve for the increase in lytic activity. The number of transformants decreased rather than increased when the cells were cultured under poor conditions or were exposed to lysozyme.

It is evident that well-transformable strains exert a high, while poorly transformable strains a low, autolytic activity. It is, however, not clear whether or not the autolytic factor enhances the development of competence.

Extracts and cells of competent *B. subtilis* 168 and SB 25 failed to influence the development of competence.

The competence of *B. subtilis* 168 developed more rapidly in the filtrate of competent than in the filtrate of incompetent cells. The difference was attributed mainly to an increased exhaustion of the competent culture filtrate.

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INCIDENCE OF TRANSMISSIBLE DRUG RESISTANCE IN PROTEUS STRAINS

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Summary. Multiple resistance to antibiotics was found in 22.6% of 124 *Proteus* strains. The presence of R factor was demonstrated in 26 strains. One type of R factor was responsible for sulphonamide, streptomycin, chloramphenicol and tetracycline resistance; the other type contained no tetracycline resistance locus.

Three strains were examined for R factor stability. Segregation of tetracycline sensitive clones was shown in one strain; in the second strain tetracycline and chloramphenicol sensitive segregants were demonstrated. No drug sensitive segregants were revealed among 503 colonies of the third strain.

The degree of R factor-mediated resistance was not higher than 100 µg/ml.

Japanese authors were the first to demonstrate the transmission by conjugation of antibiotic resistance from resistant to sensitive bacteria [1, 2]. The donor strains were, as a rule, resistant to several antibiotics, mostly to sulphonamides, streptomycin, chloramphenicol and tetracycline [3]. Donor strains resistant to other drugs have also been described [4, 5].

The transfer of resistance is characterized by a cell-to-cell transmission of resistance from the resistant to the sensitive strain. The R factor exerts two functions: it determines the resistance pattern of the strain and mediates the transfer of resistance to sensitive cells [6].

R factor-mediated antibiotic resistance is common among *Enterobacteriaceae*. In Japan 50 to 88% of multiple-resistant enteric bacteria [7], in Greece 80 to 90% of *E. coli* and *Klebsiella* strains [8], in Hungary almost 100% of resistant *Shigella* strains [9] contained R factor.

The present paper deals with the antibiotic resistance of *Proteus* strains isolated in Hungary, the transmissibility of antibiotic resistance, the degree of R factor-mediated streptomycin resistance and segregation of the R factor.

Materials and methods

Strains. The 124 *Proteus* strains used in the experiments were isolated from faeces in the Public Health Station, Szeged, and were kindly supplied by Dr. ÉVA ÁGOSTON. Primary selection of the strains was performed on the basis of Gram staining, urease reaction and lactose fermentation.

A neomycin resistant mutant of *Escherichia coli* K-12 was used as recipient in the conjugation experiment. Except neomycin susceptibility, the antibiotic resistance pattern of the mutant was the same as that of the parent strain.

Culture media. The following media were used. YP. Complete medium containing yeast extract and peptone. *M medium* [10]. NaH_2PO_4 , 6 g; K_2HPO_4 , 14 g; NH_4Cl , 2 g; NaCl , 1 g; FeCl_3 , 10 mg; distilled water, 1000 ml; pH 7.2. The medium was autoclaved at 110°C for 30 minutes. Before use the following ingredients were added to 100 ml medium: *M* MgSO_4 , 0.4 ml; 20% glucose, 4 ml; 3% agar, 100 ml. The basal medium was then supplemented with 10 $\mu\text{g/ml}$ amounts of different antibiotics or 100 $\mu\text{g/ml}$ sulphamethylthiazole. The media containing the different antibiotics were designated as SM (streptomycin), CM (chloramphenicol), TM (tetracycline) and SaM (sulphamethylthiazole).

Antibiotic sensitivity testing of *Proteus* strains was performed on YP medium with Biotest paper discs (Institute for Serobacteriological Production and Research "Human", Budapest). *E. coli* cultures were tested on SM, CM, TM and SaM media by inoculating a loopful of a dilute suspension of the strain.

Streptomycin resistance was examined by the plate dilution method on *M medium* containing 10, 20, 50 and 100 μg per ml of streptomycin.

Transfer of the *R* factor was examined as described by BÖHME [11]. Different *Proteus* strains served as donors. As a recipient, *E. coli* strain K-12 was used. Twenty-hour broth cultures of the donor and recipient strains were mixed at a proportion of 0.15 and 0.05 ml, then streaked onto agar plates. After culturing for 24 hours, growth was suspended in 3 ml of 0.15 *M* pH 7.2 phosphate buffer. Then appropriate dilutions of the dense suspension were plated onto *M* and SM agar in order to determine the number of *E. coli* colony-producing units. As *Proteus* failed to grow on *M medium*, examination of the colonies yielded a pure *E. coli* count.

Proteus strains inducing resistance in the recipient *E. coli* strain to at least two different antibiotics were regarded as *R* factor-carrying cultures. Antibiotic sensitivity testing of *E. coli* growing on SM medium after plating the donor-recipient mixture was carried out by inoculating 10 colonies into SaM, CM and TM media.

Results

Subgroup distribution of *Proteus* strains. Biochemical differentiation of the strains was performed by examining H_2S and indole production and maltose and mannitol fermentation (Table I).

Table I

Subgroup distribution of *Proteus* strains

<i>Proteus vulgaris</i>	11
<i>Proteus mirabilis</i>	109
<i>Proteus morganii</i>	3
<i>Proteus rettgeri</i>	1
Total	124

Distribution of *Proteus* strains according to antibiotic sensitivity is shown in Table II. The strains were resistant in 22.6% to sulphonamide, penicillin, streptomycin, chloramphenicol, oxytetracycline and chlortetracycline. Six of the above multiresistant strains were resistant also to neomycin. Neomycin resistance was not observed in other strains.

Table II*Distribution of Proteus strains according to antibiotic sensitivity*

	Sensitive	Moderately sensitive	Resistant
Sulphonamide	8	8	108
Penicillin	—	—	124
Streptomycin	64	16	44
Chloramphenicol	36	26	62
Oxytetracycline	1	—	123
Chlortetracycline	2	1	121
Neomycin	108	10	6

Incidence of R factor in Proteus strains. All strains were examined in conjugation experiments for the presence of R factor. It is known that the R factor may be carried by slightly resistant cultures. As the antibiotic disk method was considered suitable for the selection of highly resistant cultures only, moderately resistant and sensitive cultures were also tested.

Twenty-six out of the 124 strains carried R factor. Two of the 26 cultures were but moderately resistant to streptomycin or chloramphenicol.

It has been described that the R factor contains, in addition to the genetic determinant responsible for transmission, variable numbers of resistant loci [3]. The latter determine the drug resistance pattern of the culture.

Twenty-two out of the 26 R factor-carrying *E. coli* cultures obtained in the experiments grew in the presence of 4 different antibiotics, in other works they contained 4 resistance loci. The remaining 4 strains were sensitive to tetracycline, *i.e.* their episomes contained no tetracycline resistance determinant.

Table III*Degree of R factor-mediated streptomycin resistance in E. coli strains*

Streptomycin, $\mu\text{g/ml}$	Number of resistant strains
10	26
20	22
50	5
100	—

The degree of streptomycin resistance of the recipient strains is shown in Table III. It is seen that none of the 26 R factors transferred streptomycin resistance higher than 100 $\mu\text{g/ml}$.

Stability of the R factor was examined in 3 *E. coli* strains containing all 4 resistance loci. About 500 colony-forming units of each strain were streaked onto M medium then replica-plated by LEDERBERG's velveteen stamp method [12] to SM, SaM, CM and TM media. The results are presented in Table IV.

Table IV
Stability of R factor in E. coli strains

Designation of strain	Number of colonies				
	M	SM	SaM	CM	TM
R-7	522	522	522	517	481
R-16	498	498	498	498	497
R-21	503	503	503	503	503

All colonies of strain R-21 grew on all media. One colony of strain R-16 failed to grow on the plate containing tetracycline. The colonies of strain R-7 were sensitive to tetracycline in 8%, to chloramphenicol in 1%. Three out of the 5 chloramphenicol sensitive segregants were sensitive also to tetracycline.

Discussion

The incidence of resistant and multiresistant bacterial strains is increasing parallel with the widespread use of antibiotics. In our study, resistance of *Proteus* strains to the antibiotics usually prescribed in Hungary was examined. Our cultures were resistant at least to 3 antibiotics and 22.6% of them were resistant to streptomycin, penicillin, oxytetracycline, chlortetracycline, chloramphenicol and sulphonamide. Six of the latter cultures exhibited resistance also to neomycin, thus they were unsusceptible to all antibiotics examined.

The presence of R factor was demonstrated in 26 out of 28 multi-resistant strains. A similar incidence of R factor-carrying *Proteus* strains was described in Japan [7] and in Greece [8].

In our strains, two types of R factor were revealed. One type was responsible for sulphonamide, streptomycin, chloramphenicol and tetracycline resistance; the other type contained no tetracycline locus. Other authors described several types of R factor [7]; in the present study no efforts were made to demonstrate more types. Different types of R factor are produced as a result of segregation and recombination [3]. Segregation was observed in the present material. Strain R-7 showed the loss of resistance to two different antibiotics.

The R factor may be responsible for various degrees of antibiotic resistance [13]. In our material R factor-mediated streptomycin resistance was not higher than 100 $\mu\text{g/ml}$; this low degree of resistance might explain the frequent disagreement between laboratory sensitivity testing and therapeutic results.

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EFFICIENCY OF SMALLPOX REVACCINATION REPEATED AT THREE-YEAR INTERVALS

II. COMPARATIVE STUDY OF THE INTERNATIONAL REFERENCE STRAIN (ELSTREE) AND THE HUNGARIAN VACCINE STRAIN (BUDAPEST)

By

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Summary. Adults who had been successfully vaccinated three years earlier were revaccinated partly with the international reference strain, partly with the Budapest strain, of vaccinia virus. The two dermovaccines had the same PFU value.

Each person was vaccinated simultaneously at two sites with different doses of the same vaccine. For the site of the larger dose the take rate appeared to be independent of the strain of vaccine. In the case of the Budapest strain vaccine the take rate was hardly influenced by a fourfold diminution in the quantity of the introduced virus, whereas for the reference strain it was definitely dose-dependent.

Those who produced the same type of cutaneous reaction with different vaccines had approximately the same average neutralizing antibody titre in both the pre- and postvaccination samples. The same was valid for the haemagglutination-inhibition titres except for the three-week postvaccination ones.

The data for a younger (from 21 to 40 years) and an older (from 41 to 60 years) group of vaccinees were compared statistically. In the latter group the rate of equivocal results was higher and that of unsuccessful vaccination lower than in the former group. The antibody response was apparently identical in the two age groups.

Sixty-three hospital employees who in 1963 had successfully been revaccinated with a smallpox vaccine prepared from the Budapest strain, were revaccinated in 1966; 35 with a vaccine prepared from the international reference strain Elstree (Group Elstree), the others with a Budapest-strain vaccine (Group Budapest). The success of the vaccinations, the side reaction and the humoral antibody responses were analyzed.

Materials and methods

Materials and methods have been detailed previously [1]. In the present study two different vaccines nearly identical in PFU value were used. Each subject was scarified simultaneously at two sites, one of the scarifications having been double-cross-shaped, the other a simple one. The cutaneous reactions were evaluated according to the recommendation of the WHO [2] except that equivocal reactions were subdivided into those healing with a crust (equivocal-1) and those healing without crust (equivocal-2). The vaccinees were classified on the basis of the stronger reaction into three subgroups, *viz.*, subgroup "take" giving major reaction at one site and major, equivocal-1 or equivocal-2 reaction at the other; subgroup "equivocal" showing equivocal-1 reaction at one site and equivocal-1 or equivocal-2 reaction at the other, and subgroup "unsuccessful" with equivocal-2 reaction at both sites.

Anti-vaccinia haemagglutination-inhibiting (HI) and neutralizing antibodies were determined in pre-vaccination serum samples and in those taken 1 and 3 weeks after vaccination.

Results

I. *Success of revaccinations.* Table I shows the success of vaccination and the statistical analysis for the groups Budapest and Elstree. There were but non-significant differences between the two groups in the distribution of the vaccinees according to the type of reaction.

Table I
Results of revaccinations

Vaccine	Total number of vaccinees	Distribution of vaccinees by the vaccination result		
		take	equivocal	unsuccessful
Elstree	35	17	10	8
Budapest	28	13	5	10

Groups compared	Vaccination result	χ^2	$p\%$
Elstree	take	0.028	> 90
Budapest	equivocal	0.984	> 30
	unsuccessful	1.26	> 20

II. *Influence of the introduced amount of virus on the type of the cutaneous reaction.* In examining the influence of the dose of vaccine on the cutaneous reaction, dilution of the vaccines would have been an inadequate procedure, for three years after a successful revaccination a considerable number of major reactions cannot be expected from a diluted vaccine. It was therefore decided to quadruple the amount of virus to be introduced, by increasing the length of scarification to fourfold. In this way the effects of two different doses of each vaccine could be compared with each other and with the effects of the same doses of the other vaccine.

Table II/a

Influence of the introduced quantity of virus on the distribution by the type of cutaneous reaction

Vaccine	No. of sites of scarification	Distribution by the type of cutaneous reaction after					
		single scarification			double-cross scarification		
		major reaction	equivocal-1 reaction	equivocal-2 reaction	major reaction	equivocal-1 reaction	equivocal-2 reaction
Elstree	70	5	4	26	16	9	10
Budapest	56	9	4	15	11	6	11

Table II/b

Groups compared	Vaccine	Type of cutaneous reaction	χ^2	p%
Cutaneous reactions following single and double-cross scarification	Elstree	major	8.23	< 1
		equivocal-1	2.36	> 10
		equivocal-2	14.64	< 1
	Budapest	major	0.31	> 50
		equivocal-1	0.48	> 30
		equivocal-2	1.14	> 20

Table II/c

Groups compared	Vaccination technique	Type of cutaneous reaction	χ^2	p%
Cutaneous reactions following revaccinations with the Elstree and Budapest vaccine	single scarification	major	2.86	> 5
		equivocal-1	0.11	> 70
		equivocal-2	4.20	< 5
	double-cross scarification	major	0.26	> 50
		equivocal-1	0.15	> 50
		equivocal-2	0.80	> 30

In Table II/a the results of the double-cross and single scarifications are comparatively evaluated. Accordingly, a fourfold increase in the dose of the Elstree vaccine resulted in a significant rise in the take rate, at the expense of unsuccessful vaccinations. On the other hand, the same increase in the dose of the Budapest vaccine caused but a non-significant change in the distribution by reaction type (Table II/b).

In Table II/c the vaccination results achieved by the same doses of the two different vaccines are compared. At the site of the simple scarification equivocal-2 reactions occurred significantly more frequently in the Elstree group than in the Budapest group, whereas the difference, in the opposite sense, in the frequency of major reactions was slightly below the threshold of significance. In the case of double-cross scarification the difference between the effects of the Budapest and Elstree vaccines appeared to be far from being significant.

III. *Reactivity of the vaccines.* There was but a slight difference between the groups Budapest and Elstree in the local and general reactions following revaccination. Oedematous redness extending to the skin of the whole upper arm occurred in one case, oedematous redness exceeding 20 mm in diameter occurred in further 4 cases, all of these subjects having been revaccinated with the Elstree strain.

Axillary pain (Elstree vaccine) and general articular pain (Budapest vaccine) was registered in one case each. Headache, nausea, chills and malaise were observed in 8 and 7 subjects in the Budapest and Elstree group, respectively.

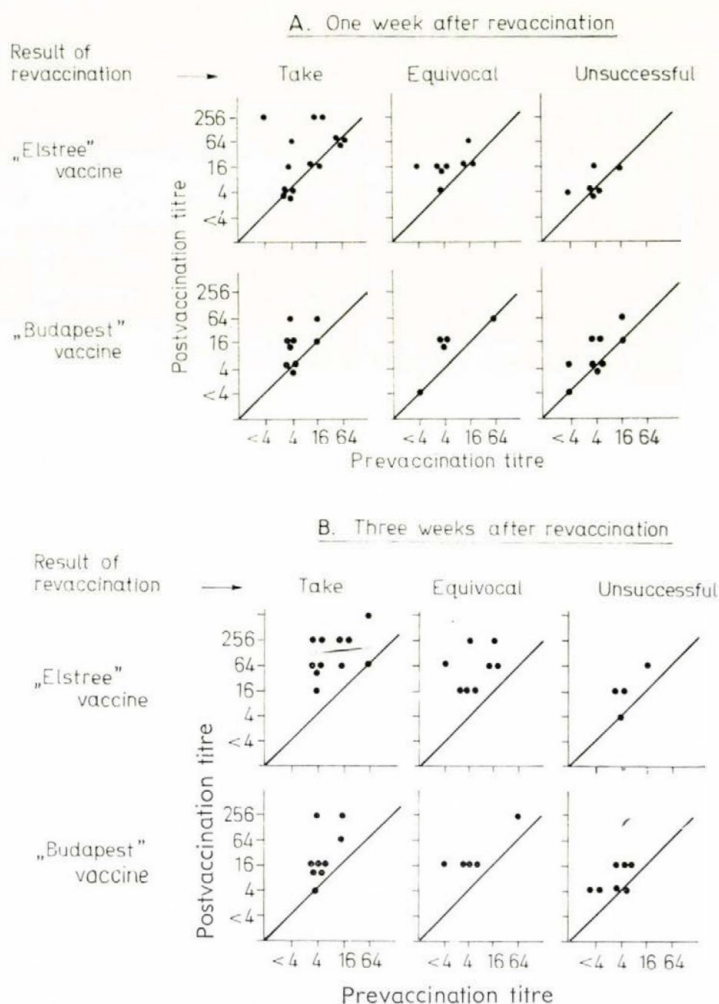


Fig. 1. Pre- and postvaccination neutralization titres

tively. Temperature between 37°C and 38°C occurred in 4 and 3 cases, respectively. Fever between 38°C and 39°C occurred in two cases, both in the group Budapest.

IV. *Pre- and postvaccination antibody titres.* Fig. 1 illustrates the changes in neutralizing antibody titres for groups Elstree and Budapest. Out of the 28 and 23 subjects vaccinated with the Elstree and Budapest vaccine respectively

equally 12 had elicited a fourfold or greater rise in the neutralization titre by the end of the first postvaccination week. Three weeks after vaccination the antibody responses were still equal in the two groups.

Fig. 2 shows the HI titres for the subgroups take. The frequency of fourfold or greater rise in HI titre as measured three weeks postvaccination appeared to be independent of the vaccine strain. The vaccinees in subgroups equivocal and unsuccessful elicited no or hardly any HI antibody response.

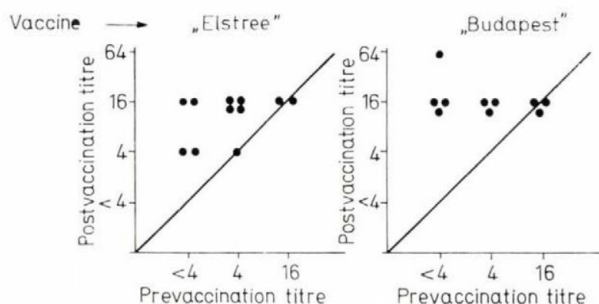


Fig. 2. Pre- and three-week postvaccination HI titres. Group take

Table III summarizes the average pre- and postvaccination titres. The differences by vaccine strain were not significant except between the subgroups take, in which the average HI titre measured three weeks after vaccination was significantly higher for the group Budapest than for the group Elstree.

V. *Correlation of antibody response and of the type of cutaneous reaction to the age of the vaccinees.* According to our previous studies [3] the success of revaccination was in the best correlation, among the factors tested, with the age of the vaccinees. Older people gave major reaction more frequently than younger ones. The more intensive reaction of older people might be attributed to some unknown age-dependent factor or to the longer time interval between the last vaccination and the revaccination.

The present vaccinees were different in age but the time that had elapsed from their last successful revaccination was equally three years. Thus, in our material the study of the age-dependence of the vaccination reaction was undisturbed by the vaccination interval.

(1) The possible age-dependence of the immunological status and reactions was studied by analyzing the following factors.

(a) Distribution of the vaccinees by the prevaccination neutralizing titre; (b) percentage of vaccinees giving rapid neutralizing antibody response; (c) distribution of the vaccinees by the neutralization titre three weeks after take and equivocal reactions; (d) distribution by the HI titre three weeks after successful revaccination.

Table III/a

Average pre- and postvaccination neutralizing and HI antibody titres of persons vaccinated with Elstree or Budapest vaccine

Vaccine	Vaccination result	Average neutralizing antibody titres			Average HI titres		
		before vaccination	1	3	before vaccination	1	3
			weeks after vaccination			weeks after vaccination	
Elstree	take	9.2	29.0	101.0	3.1	3.3	10.9
	equivocal	5.3	15.9	53.8	5.3	6.1	6.7
	unsuccessful	4.8	6.4	16.0	2.0	2.5	4.0
Budapest	take	4.5	16.3	36.8	3.5	5.3	18.4
	equivocal	5.3	11.3	27.8	2.3	2.8	4.0
	unsuccessful	4.0	7.4	7.3	3.0	3.1	3.3

Table III/b

Comparison of average neutralization titres of vaccinees with identical vaccination result

Groups compared	Vaccination result	Before vaccination			1 week			3 weeks		
					after vaccination					
		$\bar{x}_1 - \bar{x}_2$	t_x	p%	$\bar{x}_1 - \bar{x}_2$	t_x	p%	$\bar{x}_1 - \bar{x}_2$	t_x	p%
Elstree	take	1.02	$t_{21} = 1.352$	> 10	0.86	$t_{22} = 0.959$	> 30	1.71	$t_{19} = 2.060$	> 5
	equivocal	0			x			1.67	$t_{11} = 1.701$	> 10
Budapest	unsuccessful	0.25	$t_{15} = 0.381$	> 70	0.22	$t_{13} = 0.418$	> 60	1.14	$t_{10} = 1.588$	> 10

Table III/c

Comparison of average HI titres of vaccinees with identical vaccination result

Groups compared	Vaccination result	Before vaccination			1 week			3 weeks		
					after vaccination					
		$\bar{x}_1 - \bar{x}_2$	t_x	p%	$\bar{x}_1 - \bar{x}_2$	t_x	p%	$\bar{x}_1 - \bar{x}_2$	t_x	p%
Elstree	take	0.21	$t_{26} = 0.267$	> 70	0.69	$t_{22} = 1.67$	> 20	0.77	$t_{19} = 2.204$	< 5
	equivocal	1.2	$t_{13} = 1.493$	> 10	1.1	$t_{12} = 1.456$	> 10	0.75	$t_{11} = 1.102$	> 20
Budapest	unsuccessful	0.6	$t_{16} = 1.063$	> 30	0.44	$t_{13} = 0.658$	> 50	0.29	$t_{10} = 0.255$	> 80

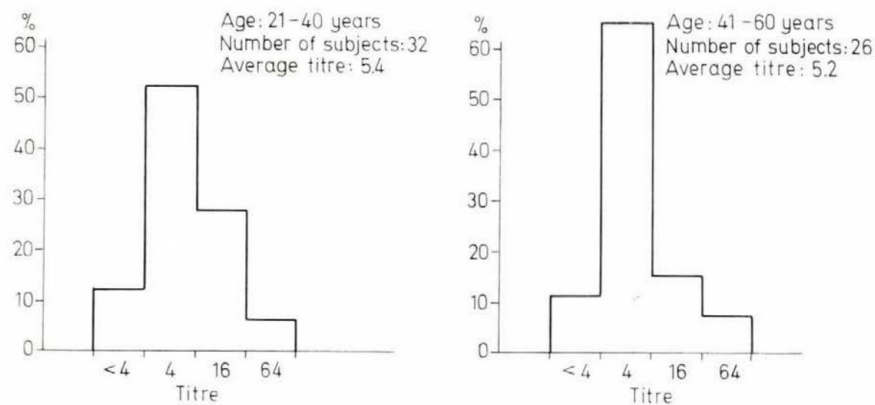


Fig. 3. Distribution by age of prevaccination neutralizing antibody titres

As to (a), (b), and (c), the neutralization titres were analyzed because these had proved to be more sensitive indicators of changes in humoral immunity than HI antibodies.

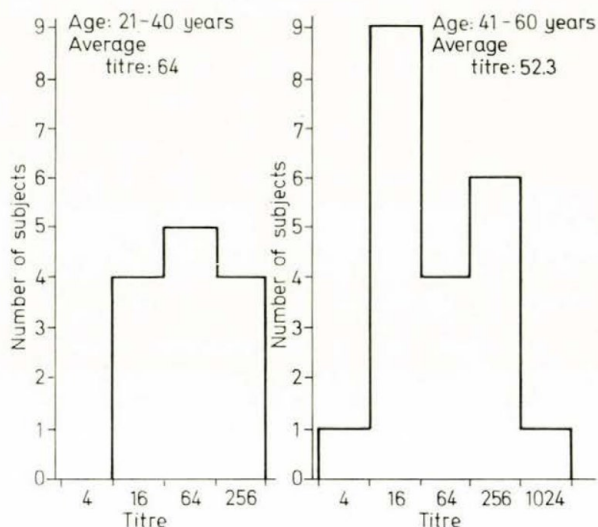


Fig. 4. Distribution of younger and older vaccinees by the neutralizing antibody titre measured three weeks after revaccination. Groups take and equivocal

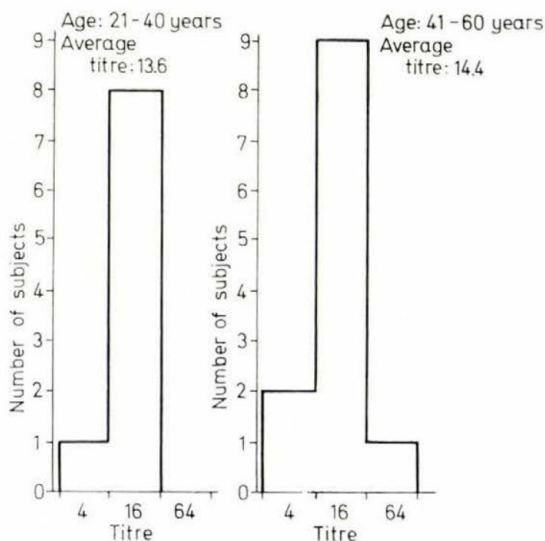


Fig. 5. Distribution of younger and older vaccinees by the HI antibody titre measured three weeks after revaccination. Group take

To analyse the present material by age, the vaccinees were divided into a younger (21-40 years) and an older (41-60 years) group.

(a) In distribution by prevaccination neutralization titre ($F_{32 \cdot 26} = 1.11$; $p > 20\%$) and in the average titre ($t_{56} = 0.257$; $p > 70\%$) there was no significant difference between the two age groups (Fig. 3).

(b) We had 16 and 20 one-week postvaccination samples from the younger and older group, respectively. Fourfold or greater increase in the neutralizing antibody titre was demonstrated in 10 and 9 cases. The difference was not significant ($\chi^2 = 0.473$; $p > 30\%$).

(c) The distribution by neutralization titres in the serum samples collected three weeks after vaccination is illustrated in Fig. 4. There was no significant difference between the two age groups either in the distribution by titre values ($F_{21 \cdot 13} = 1.695$; $p > 5\%$) or in the average titre ($t_{32} = 0.421$; $p > 60\%$).

(d) Similarly, there was no significant difference between the two age groups in the distribution by the HI titre of the serum samples taken three weeks after successful revaccination ($F_{9 \cdot 12} = 1.127$; $p > 20\%$) and in the average HI titres ($T_{19} = 0.128$; $p > 80\%$) (Fig. 5).

It may be concluded that the reactions carried out in the present study allowed no distinction between the two age groups.

(2) *Correlation between age and cutaneous reaction.* The results of revaccination in the function of age are shown in Table IV. There was no significant difference in the take rate, but the rate of vaccinations with an equivocal result was significantly higher and that of unsuccessful vaccinations significantly lower for the older group than for the younger one.

Table IV

Distribution by vaccination result of younger and older vaccinees

Age, year	Distribution of vaccinees by the vaccination result			Total No. of vaccinees
	take	equivocal	unsuccessful	
21-40	16	5	14	35
41-60	14	10	4	28

Groups compared	Vaccination result	χ^2	p%
Young	take	0.114	> 70
Old	equivocal	4.96	< 5
	unsuccessful	3.93	< 5

Discussion

The present material has been suitable for comparing two vaccines. The interval from the last successful revaccination was uniformly three years and the distribution by both age and prevaccination titre in the groups Budapest and Elstree was nearly the same. The practical absence of biases allowed to draw conclusion from a small number of observations. As regards the side effects, however, a definite conclusion could not be drawn. Local or general reactions more intensive than usual were infrequent, but it was just the small number of excessive reactions that prevented statistical evaluation.

With double-cross scarification, the efficiency of vaccination with the two different preparations of identical PFU value was nearly the same. However, in the case of single scarification the Budapest vaccine appeared to be superior to the Elstree one. Apparently, the two dose-response curves have different slopes. POLAK *et al.* [4], who compared an Elstree vaccine with a vaccine prepared from a Dutch strain, found no difference in slope and numerous investigations [4, 5, 6, 7] are suggestive of a linear relationship between PFU value and potency. These observations seem to be consistent with the principle [2] that the minimum requirement for the potency of a smallpox vaccine should be based on its pock-count on the chorio-allantoic membrane. The present observations on the other hand suggest that the pock-count may be an inadequate basis for comparison with the reference vaccine of vaccines prepared from the Budapest strain. This of course does not mean that the same would be valid if the efficiency of primary vaccination were chosen as basis of comparison. Furthermore, the fact that all the previous vaccinations of the present vaccinees had been done with the Budapest vaccine might have influenced the results. On the other hand, our suggestion that the pock-count may be an unreliable basis for the comparison of vaccines prepared from different strains is supported by the fact that in primary vaccinations a take rate of nearly 100% was achieved over numerous years with a Budapest strain vaccine of $5-10 \times 10^6$ PFU per ml value.

As regards the serological responses, the only difference between the two vaccines was that the average HI titre for the serum samples taken three weeks after vaccination was significantly higher in the Budapest group than in the Elstree group. This, being presumably in connection with the different slopes of the two dose-response curves, supports our assumption that in its tendency to multiply in the human organism the Budapest strain is superior to the reference strain.

At the beginning of our studies we had supposed that the vaccine prepared from the reference strain would prove to be superior to the Budapest vaccine. The results, however, pointed to certain advantages of the Budapest vaccine. Still, since the present group of vaccinees had a special vaccination history,

one cannot state for certain that for primary vaccination the Budapest vaccine was superior to the reference vaccine.

The uniform vaccination history of the vaccinees under study allowed to analyze the influence of age on the success of vaccination and on the serological response. Only one difference proved to be significant, *viz.*, the rate of vaccination with an equivocal result was higher for older (41—60 years of age) than for younger (21—40 years). The difference was at the expense of unsuccessful vaccinations. The take rate for, and the antibody response by, adults seemed to be independent of age.

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COMPARATIVE LABORATORY STUDY OF VACCINES PREPARED FROM THE INTERNATIONAL REFERENCE STRAIN AND FROM THE "BUDAPEST" STRAIN OF VACCINIA VIRUS

By

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Summary. Egg vaccines and bovine dermovaccines prepared from the Budapest strain of vaccinia virus and those prepared from the international reference strain (Elstree) have been studied. Rabbits were inoculated by cutaneous scarification and intradermally. LD₅₀ was determined for mice and chicken embryos and CPID₅₀ for monkey kidney cell cultures. The vaccines were of nearly the same PFU value.

The mouse LD₅₀ of the vaccine prepared from the Budapest strain was moderately, the chicken embryo LD₅₀ of the same vaccine was definitely, lower than the corresponding LD₅₀ of the reference-strain vaccine. In spite of this, the Budapest strain cannot be considered neurovirulent: the lesions caused by this strain could not be distinguished morphologically from those characteristic of the reference strain and the mouse virulence of the strain could not be enhanced by serial intracerebral passages.

The distinctness of the vaccines prepared from the two strains was reflected by the different take rates in humans.

Smallpox vaccines prepared from different strains can be distinguished in the laboratory and by human vaccination. HERZBERG [1] found vaccines more pathogenic immediately after preparation than after storage. The reduction of pathogenicity during storage could not be explained by a simultaneous decrease in infectivity. COCKBURN *et al.* [2] found that different vaccines differed in heat resistance. KUNERT and WOLFF [3] compared a Berlin vaccine and a Berne vaccine of the same PFU (pock-forming unit) value; the Berlin vaccine was more pathogenic for both rabbit and man.

In the frame of an international collaboration the reference smallpox vaccine was compared in 7 laboratories with 5 other vaccines. The results were evaluated by KRAG and BENTZON [4]. A well-defined correlation was found between the pock count on the chorio-allantoic membrane (CAM) and the titre determined by intradermal inoculation of rabbits. Some discrepancy was, however, demonstrated between these results and those obtained by other methods, such as chicken embryo LD₅₀, mouse LD₅₀, CPID₅₀, and intradermal rabbit test. It was concluded that these values are influenced by some properties of the vaccine other than the PFU.

In a previous study [5] we revaccinated humans with a vaccine prepared from the Budapest strain, the strain exclusively used in Hungary for vaccine

production, and with a vaccine of the same PFU value, prepared from the international reference strain. The take rate with the Budapest vaccine was twice that obtained with the reference-strain vaccine. If, however, the vaccine doses were quadrupled, the take rate for the reference-strain vaccine increased definitely, whereas that for the Budapest strain vaccine showed hardly any change. Similar conclusions were drawn by ERDŐS *et al.* [6] from primary vaccination of infants and 12-year-old children.

In the present study vaccines prepared from the Budapest strain have been compared in the laboratory with those prepared from the reference strain. The following values were determined: PFU on CAM, infectivity for rabbits inoculated by cutaneous scarification and for those injected intradermally, LD₅₀ for mice and chicken embryos, and CPID₅₀ for monkey kidney cell cultures.

Materials and methods

Vaccines. (1) Budapest strain vaccines. The Budapest strain had been brought to Budapest from Berlin in 1927. Since then it has been maintained in alternate calf-rabbit passages. In the initial period of the present experiments a 20% egg vaccine was used which had been prepared from the second egg passage of the dermovaccine strain. In subsequent experiments the commercial dermovaccine containing 0.5% streptomycin and 50% glycerol was applied.

(2) Reference-strain vaccines. Initially a vaccine prepared as described under (1) from the second egg passage of the dermovaccine strain was used. The vaccine applied subsequently was a bovine dermovaccine containing 0.5% streptomycin and 50% glycerol. To prepare this vaccine, the strain available in the reference vaccine had been carried over 1 passage in rabbits and 3 subsequent passages in calves.

Vaccines were stored at -20°C. A fresh ampoule was opened before each experiment.

(a) *PFU titration.* Three dilutions of a threefold dilution series of vaccine were inoculated onto the CAM of chicken embryos 11–12 days of age. The membranes were prepared as described by NADEJE [7]. Five membranes were inoculated with each dilution, 0.1 ml per membrane. The inoculated eggs were incubated at 37°C for 48 hours. Subsequently the pocks (PFU) were counted on all the mechanically uninjured CAMs and the results were averaged. This method was considered the reference method. The other results were related to the PFU.

(b) *Titration in scarified rabbit skin.* Series of tenfold dilutions were prepared from the two vaccines to be compared and 0.1 ml of each dilution was inoculated, symmetrically into the depilated and scarified skin of a rabbit. The result was read on the 6th day. The dilution next to confluency was accepted as titre.

(c) *Intradermal titration in rabbits.* 0.1 ml of each dilution of the tenfold series was inoculated intradermally in symmetrical arrangement. Results were read 72 hours later. The highest dilution causing an infiltrated redness not less than 8 mm in diameter was accepted as titre.

(d) *Determination of intracerebral mouse LD₅₀.* Albino mice not older than 24 hours were inoculated intracerebrally with 0.03 ml of virus. Five to 10 mice were inoculated with each dilution of the tenfold dilution series. Mortality on the 4–14 postinfection days was considered as specific, provided the mouse had been definitely ill (failed to move and suck and showed dyspnoea) for 1 or 2 days before death. LD₅₀ was calculated by the REED-MUENCH formula.

(e) *Determination of chicken embryo LD₅₀ by CAM inoculation.* CAMs of 5 chicken embryos 11–12 days of age were inoculated with each dilution of the tenfold series, 0.1 ml per membrane. Mortality observed in the period from 48 hours to 7 days postinoculation was considered positive, provided the CAM showed specific lesions. LD₅₀ was calculated by the REED-MUENCH formula.

(f) *Determination of CPID₅₀ in monkey kidney cell cultures.* Five primary monkey kidney cell cultures were infected with each dilution of the tenfold series, 0.1 ml per tube. After 1-hour adsorption at room temperature 1 ml PARKER'S 199 medium was added to each culture. The cultures were examined daily from 48 hours to 5 days postinfection. All cultures showing specific cytopathic (CP) lesions were regarded positive. The degree of CP lesions was neglected. CPID₅₀ was calculated by the REED-MUENCH formula.

The two vaccines were titrated simultaneously in each experiment. Furthermore, PFU titration was carried out simultaneously with each titration according to (b-e) and, if more than one month had elapsed since the last PFU titration on CAM, also with titration in tissue cultures.

Results

Infectivity titres obtained by scarification of rabbits. Parallel titrations of the two vaccines were carried out on 5 occasions, on a total of 8 rabbits. The titres are shown in column 4 of Table I. The corresponding PFU values are

Table I

Comparison of PFU values and infectivity titres obtained by cutaneous scarification of rabbits

Experiment No.	Vaccine	PFU/ml	Scarification titre/ml	PFU/ml Budapest	Scar. titre Budapest
				PFU/ml Reference	Scar. titre Reference
1	Budapest	2.4×10^7	10^4	0.6	1
	Reference	3.8×10^7	10^4		
2/1	Budapest	7.8×10^7	10^5	0.6	1
	Reference	1.3×10^8	10^5		
2/2	Budapest	7.8×10^7	10^5	0.6	1
	Reference	1.3×10^8	10^5		
3/1	Budapest	2.7×10^8	10^4	2.2	1
	Reference	1.2×10^8	10^4		
3/2	Budapest	2.7×10^8	10^5	2.2	1
	Reference	1.2×10^8	10^5		
4	Budapest	9.0×10^7	10^5	1.5	1
	Reference	6.0×10^7	10^5		
5/1	Budapest	9.5×10^7	10^5	2.0	1
	Reference	4.6×10^7	10^5		
5/2	Budapest	9.5×10^7	10^5	2.0	1
	Reference	4.6×10^7	10^5		

In experiment No. 1 egg vaccines, in the further tests bovine dermovaccines, were compared.

presented in column 3. When titration was carried out in two rabbits simultaneously (experiments 2, 3 and 5), the results were related to the same PFU value. In column 6 the quotients of the "scarification" titres for the two vaccines, in column 5 those of the PFU values, are shown. The former was constantly 1.0, the latter ranged from 0.6 to 2.2.

The lesions produced by the two vaccines were of the same appearance. Haemorrhagic, oedematous pocks surrounded by an inflammatory area were never seen on CAM. The cutaneous lesions reached their greatest size on the 5th or 6th day, the pustules did not penetrate deep. The crusts came off on the 12th to 14th days, leaving behind superficial shining scars.

Intradermal titration in rabbits. The two vaccines were titrated in parallel on 4 occasions, in a total of 6 rabbits. In columns 3 and 4 of Table II the PFU values and intradermal titres, respectively, are presented. Since the different titrations were usually carried out simultaneously, the PFU values as well as the corresponding quotients do not differ from those shown in Table I.

Table II
Comparison with PFU values of rabbit intradermal infectivity titres

Experiment No.	Vaccine	PFU/ml	Intradermal titre/ml	PFU/ml, Budapest PFU/ml, Reference	I. dermal titre, Budapest I. dermal titre, Reference
1	Budapest Reference	2.4×10^7 3.8×10^7	10^5 10^4	0.6	10
2/1	Budapest Reference	7.8×10^7 1.3×10^8	10^5 10^5	0.6	1
2/2	Budapest Reference	7.8×10^7 1.3×10^8	10^4 10^4	0.6	1
3/1	Budapest Reference	2.7×10^8 1.2×10^8	10^5 10^5	2.2	1
3/2	Budapest Reference	2.7×10^8 1.2×10^8	10^5 10^5	2.2	1
4	Budapest Reference	9×10^7 6×10^7	10^5 10^5	1.5	1

See footnote to Table I.

Table III
Comparison with PFU values of mouse intracerebral LD₅₀ values

Experiment No.	Vaccine	PFU/ml	LD ₅₀ /ml	PFU/ml, Budapest PFU/ml, Reference	LD ₅₀ /ml, Budapest LD ₅₀ /ml, Reference
1	Budapest Reference	7.8×10^7 1.3×10^8	3.3×10^8 6.8×10^7	0.6	4.8
2	Budapest Reference	2.7×10^8 1.2×10^8	2.1×10^8 3.9×10^7	2.2	5.3
3	Budapest Reference	2.1×10^7 2.1×10^7	7.4×10^7 3.9×10^6	1.0	18.9
4	Budapest Reference	9.5×10^7 4.6×10^7	1.3×10^8 5.8×10^7	2.0	2.2
5	Budapest Reference	9.7×10^7 4.6×10^7	1.7×10^8 1.3×10^7	2.1	13.1

In experiment No. 4 egg vaccines, in the other tests bovine dermovaccines, were compared.

The quotient of the intradermal titres was 1.0 on 5 occasions, whereas the titration on one rabbit resulted in a quotient as high as 10, presumably due to technical error. There was no gross difference between the lesions caused by the two vaccines, and their appearance in time was also similar.

Mouse LD₅₀ values (Table III). Calculations were performed as described above. In contrast to the approximately identical PFU values, the LD₅₀ of the reference-strain vaccine was 2.2–18.9 times higher than that of the Budapest-strain vaccine. Accordingly, the Budapest strain is slightly more virulent for the mouse than the reference strain.

Table IV
Comparison with PFU values of chicken embryo LD₅₀ values

Experiment No.	Vaccine	PFU/ml	LD ₅₀ /ml	PFU/ml Budapest PFU/ml Reference	LD ₅₀ /ml Budapest LD ₅₀ /ml Reference
1	Budapest	3.2×10^7	5.0×10^7	1.4	16
	Reference	2.3×10^7	3.2×10^6		
2	Budapest	2.07×10^7	1.0×10^8	0.6	62.5
	Reference	3.9×10^7	1.6×10^6		
3	Budapest	2.1×10^7	2.0×10^7	1	80
	Reference	2.1×10^7	2.5×10^5		
4	Budapest	2.7×10^8	1.0×10^7	2.2	40
	Reference	1.2×10^8	2.5×10^5		
5	Budapest	7.8×10^7	1.0×10^8	0.6	40
	Reference	1.3×10^8	2.5×10^6		

In experiments No. 1, 2 and 3 egg vaccines, in tests No. 4 and 5 bovine dermovaccines, were compared.

Table V
Comparison with PFU values of CPID₅₀ values

Experiment No.	Vaccine	PFU/ml	CPID ₅₀ /ml	PFU/ml Budapest PFU/ml Reference	CPID ₅₀ /ml Budapest CPID ₅₀ /ml Reference
1	Budapest	2.7×10^8	4×10^7	2.2	2
	Reference	1.2×10^8	2×10^7		
2	Budapest	1.4×10^8	2×10^8	2.2	1.5
	Reference	6.2×10^7	1.3×10^8		
3	Budapest	N. T.	3.2×10^8	—	2.4
	Reference	N. T.	1.3×10^8		
4	Budapest	N. T.	3.2×10^8	—	5
	Reference	N. T.	6.3×10^7		
5	Budapest	1×10^8	2×10^8	0.5	1.5
	Reference	1.8×10^8	1.3×10^8		

Bovine dermovaccines were compared throughout.
N. T. = not tested.

Chicken embryo LD₅₀ values (Table IV). The LD₅₀ of the Budapest strain vaccine was 16 to 80 times smaller than that of the reference-strain vaccine, indicating that the Budapest strain is definitely more virulent for the chicken embryo than the reference strain.

CPID₅₀ values for monkey kidney cell cultures (Table V). The CPID₅₀ values of the two vaccines were approximately the same. The PFU and CPID₅₀ values agreed well, except for one experiment.

Discussion

We attempted to characterize smallpox vaccines by comparing their infectivity titres as obtained by different methods. These investigations were justified by the observation that in the course of the characterization of the reference vaccine this method had proved to be suitable for the demonstration of distinct properties of different vaccines [4]. The result has fulfilled expectations. The Budapest strain, though it induced lesions morphologically identical with those caused by the reference-strain vaccine, proved to be definitely more pathogenic for the chicken embryo, and slightly more pathogenic for the mouse, than the reference strain. Pock counting, being the officially accepted method of smallpox vaccine standardization, was chosen as reference method. In the course of repeated tests the PFU value of the Budapest-strain vaccine was never higher than twice that of the reference-strain vaccine. In contrast, its mouse LD₅₀ was 2–19 times and its chicken embryo LD₅₀ was 16–80 times smaller than the respective doses of the reference-strain vaccine. It should be emphasized that the embryo's sensitivity to the lethal effect of the vaccine was fluctuating indicating that the chick-embryo virulence of a vaccine cannot be determined accurately without the simultaneous titration of the reference vaccine.

In spite of its moderately higher mouse-virulence the Budapest strain cannot be considered neuro-virulent. It induced lesions characteristic of neuro-virulent strains neither on CAM nor in the rabbit skin. Furthermore, we failed to enhance the neurovirulence of the strain even by 77 successive intracerebral passages in baby mice [9], nor did HOLLÓS [10] succeed in enhancing its neurovirulence by 101 serial passages in adult mice.

The question arises of the significance of the enhanced chicken embryo and mouse-virulence of the Budapest strain. It has been mentioned in the introduction that (i) the Budapest-strain vaccine produced a higher take rate than the reference-strain vaccine of the same PFU value when it was used for revaccination of humans, and (ii) that raising of the introduced amount of virus resulted in a significant rise of the take rate with the reference-strain vaccine but in hardly any change with the Budapest-strain vaccine. The differences observed in the laboratory may presumably be related to such phenomena.

It should be stressed that we have observed in the effect of vaccines differences which did not manifest themselves in the PFU value. It would therefore be reasonable to determine, in addition to their usually examined characteristics, the chicken-embryo-virulence of the smallpox vaccines in terms of the reference vaccine.

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THE LIPID COMPOSITION OF PATHOGENIC AND SAPROPHYTIC MYCOBACTERIA

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Summary. Thin layer chromatography of *Mycobacterium tuberculosis* and *M. bovis* showed that the two organisms contained identical lipid fractions. *M. avium* strains differed from mammalian mycobacteria in containing an additional component separating between waxes and triglycerides. No detectable amounts of free fatty acids were revealed in saprophytic strains.

Gas chromatography showed the presence of C_{12} to C_{22} fatty acids in *M. tuberculosis* and *M. bovis*. The only difference between the two organisms was that *M. bovis* strains contained a $C_{18:2}$ fatty acid not present in *M. tuberculosis*. No unsaturated fatty acids were shown in strains BCG and AN₅. In *M. avium* C_{12} to C_{18} fatty acids were detected; C_{18} fatty acids were represented in this organism by $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ compounds. The unsaturated fatty acid content was lower in saprophytic than in pathogenic strains.

Lipid haptens in *M. tuberculosis*, *M. bovis* and *M. avium* gave immunological cross reactions with one another, but did not react with immune sera prepared against saprophytic mycobacteria.

Pathogenic and saprophytic mycobacteria are characterized by special structural components consisting of lipids and waxes. The biological differences between mycobacteria and other microorganisms may be attributed to the presence of the said components in all mycobacterial species.

The structural and functional role of mycobacterial lipids, although an extensive research work has been carried out on this subject, is in many respects unknown. It has been shown that acid and alcohol fastness of mycobacteria is due, in addition to the role of numerous complex constituents such as lipids, fatty acids, waxes and large molecule alcohols, to non-esterified mycolic acid [3].

Cord formation by virulent strains is also associated with lipids. The substance responsible for this phenomenon has been isolated and shown to be a toxic glycolipid [6, 9].

ANDERSON [2] reported on the isolation of several lipid fractions characteristic of mycobacteria. AEBI, ASSELINEAU and LEDERER [1] differentiated several lipid and wax substances on the basis of solubility. MEYER [8] demonstrated that *M. tuberculosis* contained lipid haptens. The presence of these substances in this organism as well as in *M. bovis* has been confirmed [13]. Other workers elucidated the antigenic properties of phosphatides [14]. Chromatographic and spectrophotometric methods opened the way for the detection of characteristic lipid substances in pathogenic and saprophytic mycobacteria [11].

Some authors were able to distinguish between certain mycobacterial species on the basis of lipid composition [7, 10].

The present paper gives an account of the lipid components in pathogenic and saprophytic mycobacteria.

Materials and methods

Strains. Lipid extracts were prepared from the following strains: *M. tuberculosis* H₃₇ and E; *M. bovis* Rv, AN₅, Gräub and BCG; *M. avium* GH, Bp, Ch, BpZ; *M. smegmatis*, *M. minetti* and *M. giae*.

Lipid substances were extracted with chloroform : methanol (1 : 1) as described by FOLCH [4]. The extract was concentrated by evaporation then a petroleum ether solution containing 1000 µg lipid per ml was prepared. Thin layer chromatography was performed in silica-G gel as described by STAHL [12] using petroleum ether : ethyl ether : glacial acetic acid (90 : 10 : 1) as a solvent. The lipids were detected in iodine vapour.

Fatty acid composition of the extract was analysed gas-chromatographically after saponification in alcohol.

For immunochromatography the extracted lipids were concentrated by evaporation and dissolved in pyridine. Then an aqueous solution was prepared with cholesterol. A 0.2 ml portion of the solution obtained in this manner was incubated with 0.1 ml immune serum for 30 minutes at 32°C. Chromatography was performed by the ascending technique using Schleicher-Schuell 2024/A paper impregnated with a 5 per cent aqueous solution of Tween-80. A pH 9 buffer solution served as a solvent.

Results

1. *Thin layer chromatography.* On chromatograms of *M. bovis* strains, in addition to phospholipids which failed to run, in the order of their R_f value the following components had separated: monoglycerides, sterines, diglycerides,

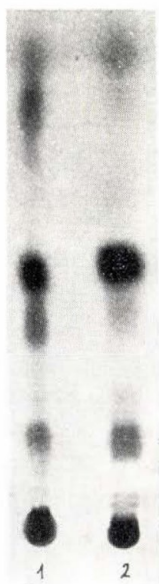


Fig. 1. Thin layer chromatogram. 1 = *M. bovis* Rv; 2 = *M. tuberculosis* H₃₇

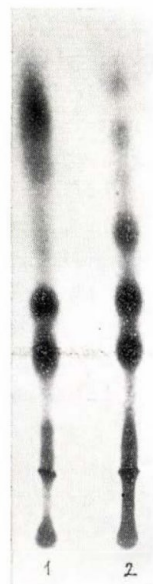


Fig. 2. Thin layer chromatogram. 1 = *M. bovis* Gräub; 2 = *M. avium* GH

free fatty acids, waxes, triglycerides, sterine esters. All tested *M. bovis* strains behaved uniformly.

M. tuberculosis strains contained the same lipids as *M. bovis* cultures. Some differences were noted between the two species in the quantitative distribution of lipid components (Fig. 1).

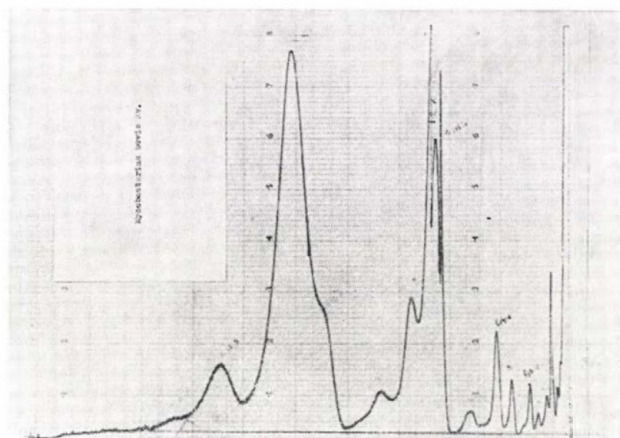


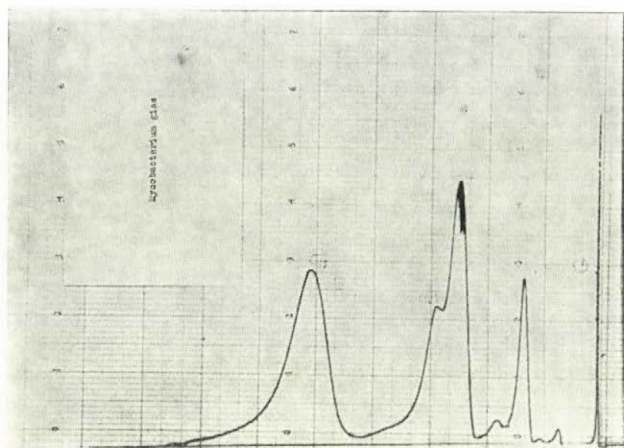
Fig. 3. Gas chromatogram. *M. bovis* Rv



Fig. 4. Gas chromatogram. *M. avium* GH

All *M. avium* strains were characterized by identical lipid patterns. They differed from *M. tuberculosis* and *M. bovis* strains in containing an additional component appearing between triglycerides and waxes (Fig. 2).

The lipid composition of saprophytic mycobacteria differed considerably from the above pattern. No fatty acids could be demonstrated in *M. smegmatis*, *M. minetti* and *M. giae*.

Fig. 5. Gas chromatogram, *M. giae*

	M.tub.				M.bovis				BCG				AN ₅				M.avium				M.giae			
	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
C ₁₀																								
C ₁₂																								
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Fig. 6. Fatty acid analysis of various mycobacteria. 0 = saturated fatty acids; 1, 2, 3 = fatty acids with the corresponding number of unsaturated bonds

2. *Gas chromatography.* In *M. tuberculosis* and *M. bovis* strains fatty acids containing 12 to 22 carbon atoms were present. Both species contained C_{16:1} and C_{18:1} fatty acids; *M. bovis* contained in addition a C_{18:2} compound (Fig. 3). In strains BCG and AN₅ no unsaturated fatty acids were detected.

M. avium strains were characterized by lower fatty acids. In addition to $C_{18:1}$ and $C_{18:2}$ the cultures contained an unsaturated fatty acid ($C_{18:3}$) not present in other mycobacteria (Fig. 4).

In saprophytic strains C_{14} to C_{18} fatty acids were shown (Fig. 5).

The result of fatty acid analysis is summarized in Fig. 6.



Fig. 7. Immunochromatogram. 1 = serum control; 2 = *M. bovis* Rv lipid hapten + *M. bovis* serum; 3 = *M. bovis* Rv lipid hapten + *M. smegmatis* serum; 4 = *M. bovis* Rv lipid hapten + *M. avium* serum; 5 = antigen control

3. *Immunochromatography*. Lipid haptens were demonstrated in *M. tuberculosis*, *M. bovis* and *M. avium* strains. The lipid haptens of *M. tuberculosis* and *M. bovis* reacted with *M. avium* immune serum (Fig. 7) and *M. avium* lipid haptens with sera for the two mammalian mycobacteria.

No lipid haptens were detected in saprophytic strains. Immune sera prepared with these organisms were inactive against the lipid haptens of *M. tuberculosis*, *M. bovis* and *M. avium*.

Discussion

Due to their lipid and wax content, pathogenic and saprophytic mycobacteria differ in biological properties from other microorganisms. These substances are therefore important partly for the exact identification of various bacterial species, partly for the elucidation of the nature of cross-reactions obtained in serological diagnostic procedures.

The purpose of the lipid analysis presented in this paper was to compare pathogenic and saprophytic mycobacteria in respect to their lipid composition and fatty acid content. The nature of serologically reactive hapten components of lipids was another problem investigated in this study.

In *M. tuberculosis* and *M. bovis* thin layer chromatography showed the components to be identical in number and R_f value. The two organisms differed only in the quantitative distribution of these components.

M. avium strains differed from mammalian mycobacteria in containing a component separating between waxes and triglycerides.

The lipid extract of saprophytic mycobacteria contains no fatty acids.

M. tuberculosis and *M. bovis* contained fatty acids with 12 to 22 carbon atoms. The latter organism differed from the former in containing a C_{18} fatty acid with two unsaturated bonds. Fatty acids consisting of more than 22 carbon atoms were not demonstrated.

Strains BCG and AN₅ contained no unsaturated fatty acids.

In *M. avium* strains the fatty acids were characterized by 18 or less carbon atoms; $C_{18:3}$ fatty acid was shown in addition to $C_{18:1}$ and $C_{18:2}$.

In saprophytic mycobacteria C_{12} to C_{18} fatty acids were demonstrated; these organisms contained unsaturated fatty acids in considerable smaller amounts than *M. avium* cultures.

Mammalian strains were composed mainly of higher fatty acids and saprophytic bacteria contained lower fatty acids in larger amounts; *M. avium* occupied an intermediary position in this respect (Fig. 6).

Lipid haptens were detected only in *M. tuberculosis*, *M. bovis* and *M. avium*. The lipid haptens were not species-specific factors as they reacted not only in the homologous systems but, with the exception of saprophytic strains, also in heterologous systems.

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INVESTIGATIONS INTO THE TESTING OF METISAZONE PREPARATIONS

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Summary. (1) Toxicity of two metisazone preparations was examined in chick embryo fibroblast monolayers, 12-day-old chick embryos and intraperitoneally injected mice. As toxic phenomena, disturbances in faecal discharge, blood in faeces, and a bright yellow coloration of the urine due to a substance devoid of antiviral effect were observed.

(2) The potency test on the chorio-allantoic membrane proved superior in accuracy to that in chick embryo fibroblast monolayers.

In mice three doses of both preparations were tested against two doses of the non-neurovirulent Budapest strain of vaccinia virus. Potency was calculated from the degree of inhibition of the virus multiplication in the mouse brain. The accuracy of the method may be satisfactory, but it is too laborious for routine use.

(3) The ratio of the largest nontoxic dose to the smallest dose eliciting a well-defined antiviral effect was found between 1 : 1 and 4 : 1 in chick embryo fibroblast monolayers, between 6 : 1 and 12 : 1 in the chorio-allantoic membrane and about 16 : 1 in the mouse.

(4) It seems to be necessary to standardize potency test for antiviral drugs. In the case of the vaccinia virus the potency test on the chorio-allantoic membrane appears to be standardizable.

(5) The two metisazone preparations proved to be approximately identical in both toxicity and potency.

After the first successful antiviral experiment with benzaldehyde thiosemicarbazone [1] 13 years had to elapse and a series of studies [2, 3, 4] had to be carried out until the first successful field trial [5] was carried out with Marboran^R. The slow progress in this field may be attributed to the lack of confidence in the future of viral chemoprophylaxis on the one hand and to the lack of adequate in vitro [6, 7, 8, 10] and in vivo [9, 10] potency tests on the other.

As for metisazone itself, there are difficulties in the potency tests in vitro [11—15], but even comparative assays in vivo are not quite satisfactory [16]. Experimental data on the toxicity of the drug are lacking. In clinical reports its toxic character has often been mentioned [17—21]. Some authors found the drug intolerable [17—19] while according to others [20—21] its toxicity is negligible.

In an attempt to study the effect of metisazone by various biological and morphological methods, we have compared two such preparations. In this paper the relative value of the applied methods will be discussed.

Materials and methods

Virus strain. The Budapest strain available in the Hungarian dermovaccine was adapted to embryonated eggs by 10 serial passages on chorio-allantoic membrane (CAM). For passages in embryonated eggs and cell cultures 20% suspensions were prepared in skimmed milk from CAM infected with the adapted virus. The suspensions were distributed in ampoules and stored at -20°C . A new ampoule was opened before each titration [22].

Attempts were made to select a neurovirulent variant from the Budapest strain. For this purpose mice weighing from 12 to 14 g were inoculated intracerebrally each with 0.03 ml of a 10% CAM homogenate. After 96 hours 4–6 mice were killed, their brains were pooled and from the 10% brain suspension a tenfold dilution series up to the 10^{-5} limit dilution was prepared. Then 4 mice were inoculated with each dilution intracerebrally. When a mouse had died, its brain was titrated in the same manner. Mortality attributable to virus infection was never observed. This experiment was repeated several times without success.

Subsequently, it was attempted to adapt the Budapest strain to mice by 101 serial intracerebral passages in mice weighing from 12 to 14 g. The virus multiplied in the mouse brain, but did not kill the mice. In the following experiments fresh brain suspensions from the 40th to 50th brain passages of the virus were used.

Metisazone preparations. Marboran^R and a Hungarian preparation (in the following HIBT) were used. Marboran^R was kindly supplied by DR. D. J. BAUER, London, and the Hungarian preparation by the Chemical Works of Gedeon Richter Ltd., Budapest.

Inoculation of mice. For toxicity tests albino mice from the breed of this Institute, weighing 16–18 g each, were inoculated with various doses of Marboran^R or HIBT suspended in 0.5 ml of saline. The controls were injected with 0.5 ml of saline.

Vaccinia virus was inoculated in a volume of 0.03 ml into the right hemisphere with a 20-gauge cannula.

To follow virus multiplication, 4 mice were decapitated at intervals, their brains were pooled, homogenized with quartz sand in a mortar and suspended in approximately 9 parts of saline. The suspension thus obtained was centrifuged at 3000 r. p. m. for 20 minutes. The sediment was discharged.

Inoculation of eggs. Eggs of white Leghorn hens were pre-incubated for 11–13 days, then 0.2 ml of appropriate dilutions of Marboran^R or HIBT were inoculated into the yolk sac. Vaccinia virus was inoculated onto the CAM by the pseudo-air-sac technique recommended by NADEJE *et al.* [23]. Four eggs were inoculated with each dilution of a tenfold series, 0.1 ml per egg. The eggs were re-incubated for 48 hours at 37°C . The pocks were counted on a watch-glass, against a dark background.

Cell cultures. POSTLETHWAITE's [24, 25] method was used with a slight modification. Suspensions of 3×10^5 cells per ml were prepared from 10-day-old chick embryos by digestion in 0.25% trypsin solution. Ten ml of the cell suspension was placed in a Petri dish. The growth medium was 15% bovine serum — 85% Hanks' balanced salt solution enriched with 0.5% lactalbumin hydrolysate. The Petri dishes were incubated in a tightly closed plexiglass box, each in a separate compartment, without introducing CO_2 into the atmosphere. Confluent monolayers were obtained in 48 hours. These were rinsed twice with PBS. The virus inoculum was contained in 1 ml Parker's medium 199. After a 30 minute adsorption period, 9 ml Parker's 199, with or without antiviral substance, was added.

The cultures were re-incubated at 37°C for 36 hours in the plexiglass box, then fixed for 2 hours in 5% formalin, rinsed with tap water, stained for 15 minutes with 1 : 10 diluted Giemsa solution and again rinsed with tap water. Plaques were counted under a magnifying glass.

Results

I. Toxicity tests

1. **Toxicity tests in chick embryo monolayers.** Forty, 20, 10, 5, 2.5, 1.25 or $0.625 \mu\text{mol}$ of Marboran^R or HIBT were added to each monolayer in 9 ml medium. Three cultures were treated with each dilution. After 48 hours the cultures were inspected with the naked eye, then examined under a magnification of 100–250 first in the native state and then after staining.

The effect of the two preparations was approximately identical. Under the effect of 40 μmol 10–50% of the apparently intact monolayers detached from the glass when rinsed. In the presence of 20 μmol , scattered gaps about 1 mm in diameter were seen. Ten μmol or less caused no macroscopic change. In the stained preparation pre-treated with 40 μmol , 50–80% of the cells appeared intact, the others were rounded and showed toxic granulation. In the monolayers treated with 20 μmol , toxic granulation was visible in 30–40% of the cells. Ten μmol or less of the drug caused no microscopically visible lesions.

2. *Toxicity test in the embryonated egg.* (a) *Inoculation into the pseudo-air-sac.* Four eggs were inoculated with each dilution of a halving series from 40 μmol to 0.156 μmol in the inoculum. The eggs were incubated at 37°C and candled daily; all the embryos died in 72 hours.

(b) *Allantoic inoculation.* The quantity introduced was the same as above. The eggs were incubated at 37°C. All the embryos died in 72 hours.

(c) *Inoculation into the yolk sac.* The same quantities were introduced. In general, the embryos died in 48 hours if the drug had passed through the hole made with the cannula in the yolk sac, and thus reached any other embryonic membrane. The mortality in the first 72 hours of the embryos inoculated with 40 or 20 μmol of the drug varied, but there was no appreciable difference in toxicity between the two preparations. Embryos injected with 40 μmol survived 96 or 120 hours only exceptionally. Of those injected with 20 μmol about 60% survived these periods. The embryos injected with smaller doses survived.

3. *Toxicity tests in mice.* Twenty mice were injected intraperitoneally with each dilution. In the first series of experiments the effect of a single dose was examined.

The mice which had received 80 μmol of the drug immediately showed toxic symptoms such as a ruffled fur, dyspnoea, failure to move, refusal of food, and died in a few hours. Those having received 40 or 20 μmol also showed a ruffled fur, and moved little, but these symptoms improved in a few hours. Injection of 10, 5 or 2.5 μmol of the drug inhibited the animals' activity for 30–90 minutes. Animals treated with smaller doses displayed no symptoms. There was no difference in effect between the two preparations.

The symptoms were attributed to the physical character, *viz.*, the coarse granules of the drug. Significant unabsorbed amounts were found in the peritoneal cavity as late as 6 days after the injection, even if the dose was not larger than 2.5 μmol . Considering that the absorbed portion of the drug was small we attributed the symptoms first of all to a peritoneal excitation.

In the second series of experiments six groups, each consisting of 20 mice, were injected intraperitoneally with a daily dose of 40, 10 or 2.5 μmol of Marboran^R or HIBT per mouse. Seven mice died among those treated with Marboran^R and 7 among those treated with HIBT. Mortality in 120 hours was 4, 1 and 2, in the order of the decreasing dose of Marboran^R, and 4, 3 and 0 in the

order of the decreasing dose of HIBT. This mortality rate did not express the toxicity of the drug, since in other experiments none of the mice having received less than 40 μmol died and mortality was minimal even with 40 μmol .

From the second day on the mice that had received 40 μmol of the drug discharged urine bright yellow in colour and the surface of the faeces of some of these mice was tinged with blood. On the third day the faeces of all the mice contained blood and on the 4th day most of the mice were constipated. After the 6th day the faeces became normal. BAUER [27] attributed such defaecation disturbances to intestinal obstruction.

Embryonated eggs were injected into each yolk sac with 0.2 ml of the coloured urine. The CAM of the same eggs was simultaneously infected with 34 PFU of vaccinia virus. The coloured urine failed to reduce the poek count. We agree with RAO *et al.* [26] in attributing the bright yellow coloration to an ineffective decomposition product.

II. Potency tests

Potency tests in cell cultures. The antiviral effects exerted by 40, 10, 2.5 and 0.625 μmol doses were examined against 17, 34 and 68 PFU of virus. The degree of inhibition was expressed in percentage plaque reduction (Table I).

Table I

Inhibition of vaccinia virus multiplication in chick embryo fibroblast monolayers expressed in per cent plaque reduction

PFU* of virus per cell culture	Concentration of metisazone in medium; μmol	Inhibition, per cent	
		Marboran ^R	HIBT
17	40	88	75
	10	94	92
	2.5	82	65
	0.625	—	—
34	40	77	77
	10	54	72
	2.5	9	39
	0.625	—	—
68	40	80	40
	10	20	40
	2.5	20	—
	0.625	—	—

* PFU = tissue-culture plaque-forming unit.

The wide scatter of the values was conspicuous. Repeated experiments resulted in similarly inconsistent results, probably due to the fact that the drug, being insoluble, is not spread uniformly on the surface of the monolayer. The virus titrations, on the other hand, always gave well-reproducible results.

Detachment of the monolayers inoculated with 40 μmol of the drug per dish interfered with reading the result; 0.625 μmol was not inhibitory at all. Fifty per cent plaque reduction took place between 2.5 and 0.625 μmol when 17 PFU of virus were contained in the inoculum, and between 10.0 and 2.5 μmol when 34 PFU were present. In the presence of 68 PFU the 50% plaque reduction took place between 40.0 and 10.0 μmol for Marboran^R, whereas the plaque reduction caused by 40 μmol of HIBT remained under 50%.

The chick embryo fibroblast cultures proved to be 5 to 10 times less sensitive to the vaccinia virus than the CAM, *i.e.*, 1 PFU for these cell cultures equalled 5 to 10 pock-forming units for the CAM. Calculated from the smallest innocuous dose (= 10 μmol) and the dose causing 50% plaque reduction in the presence of 34 PFU of virus (10–2.5 μmol) the "therapeutic" index may be estimated between 1 and 4.

2. *Potency test in embryonated eggs.* Eleven-day eggs were inoculated into the yolk sac with 40, 10 2.5 or 0.625 μmol of Marboran^R or HIBT suspended in 0.2 ml of saline. The embryos that had died in 24 hours were discarded. The survivors were inoculated on the CAM with 34, 68 or 136 egg-PFU of virus. At least 4 eggs received each combination of virus and drug. The experiment was evaluated after 48-hour incubation. The pock reduction is presented in Table II. Forty μmol of each preparation caused 100% reduction equally from 34 PFU and 68 PFU. When more of the virus or less of the drug was administered, the inhibition was partial. There was no contradiction in the results and the experiment was well reproducible. If in the repeated experiment the virus dose was not exactly the same, the reduction values were also somewhat different, but within the same experiment the data for the two preparations were consistent. In our opinion, for standardization of metisazone preparations titration on CAM may be a suitable method. The 50% pock reduction was at 0.625 μmol (34 PFU) and 1.68 μmol (68 PFU), respectively, for Marboran^R and 1.13 μmol and 1.66 μmol , respectively, for HIBT. The 50% pock reducing doses against 136 PFU of virus could not be compared because the reduction by 10 μmol Marboran^R was lacking.

Considering that 1.66–1.68 μmol of metisazone reduced the pock count by 50%, the therapeutic index was at least 6. If the mortality of the eggs given 20 μmol is considered nonspecific, the therapeutic index may be estimated at 12.

Potency test in mice. Virus multiplication in the mouse brain was followed after infection as shown in Tables III and IV. At the given times 4 mice in each group were killed, their brains were pooled and the pools were titrated on CAM.

Table II

Inhibition of vaccina virus multiplication in embryonated egg CAM, expressed in per cent pock reduction

PFU* of virus per egg	Dose of metisazone per egg μmol	Inhibition, per cent	
		Marboran ^R	HIBT
34	40	100	100
	10	81	89
	2.5	—	—
	0.625	50	45
68	40	100	100
	10	—	74
	2.5	67	62
	0.625	28	35
136	40	69	—
	10	—	60
	2.5	41	33
	0.625	0	0

PFU = embryonated egg pock-forming unit.

Table III

Inhibition of virus multiplication in mouse brain

Intraperitoneal injection 3 times with	PFU $\times 10^3$ per 100 mg brain tissue				Inhibition in per cent of the control			
	48	96	144	192	48	96	144	192
	hours after infection				hours after infection			
0.25 ml saline	2.33	18.9	76	1.89				
<i>Marboran</i> ^R								
40 μmol	0.10	3.2	4.7	0.32	96	84	94	83
10 μmol	0.08	4.2	10.0	3.20	97	78	88	0
2.5 μmol	0.10	10.0	20.0	2.0	96	47	75	0
<i>HIBT</i>								
40 μmol	0.10	1.0	4.7	0.63	96	95	94	67
10 μmol	0.63	4.2	10.0	6.3	72	76	88	0
2.5 mol μ	0.10	1.0	16.0	1.0	96	95	79	47

Inoculum: 10^2 PFU per mouse.

The mice received three doses, at 1, 24 and 48 hours after infection.

Table IV
Inhibition of virus multiplication in mouse brain

Intraperitoneal injection 3 times with	PFU $\times 10^4$ per 100 mg brain tissue						Inhibition in per cent of the control					
	48	72	96	120	168	192	48	72	96	120	168	192
	hours after infection						hours after infection					
0.25 ml saline	10.0	42.1	63.2	34.7	5.7	0.42						
<i>Marboran</i> ^R												
40 μ mol	0.94	6.0	11.0	3.2	1.0	0.10	91	86	83	91	83	77
10 μ mol	1.0	14.8	14.5	31.6	4.2	0.10	90	66	78	10	27	77
2.5 μ mol	9.2	8.5	38.5	27.0	6.3	0.32	8	79	39	27	$\emptyset$	25
<i>HIBT</i>												
40 μ mol	5.7	9.8	7.6	3.2	1.0	0.32	43	76	88	91	83	25
10 μ mol	1.6	6.3	7.3	10.0	1.0	0.31	84	84	87	72	83	25
2.5 μ mol	2.0	29.0	10.0	10.0	3.1	0.16	80	33	85	72	45	62

Inoculum: 3.16×10^4 PFU per mouse.

The mice received three doses, at 1, 24 and 48 hours after infection.

The dose of virus was 10^2 PFU and 3.16×10^4 PFU as titrated on CAM in the experiment presented in Tables III and IV, respectively. In the brain of mice which had received the smaller inoculum without the drug, the peak virus titre was reached by the 144th hour, and in those infected with the larger dose, by the 96th hour. The rest of the animals survived the 10-day period of observation. We failed in recovering virus from the blood, lungs and spleen of the animals infected with the larger dose of virus.

From the data presented in Tables III and IV it may be concluded that the inhibition of virus multiplication in the mouse brain was well-defined and in this respect, too, the two preparations were approximately equally effective.

Against the smaller dose of virus, 2.5 μ mol of the drug was approximately as effective as the larger doses were. Against the larger inoculum the 10 and 2.5 μ mol doses were somewhat less effective than 40 μ mol, and inhibition by the 10 μ mol and 2.5 μ mol doses of *Marboran*^R was less pronounced than the inhibition exerted by the corresponding doses of *HIBT*. However, the differences in this respect were not consistent enough to conclude that *HIBT* was superior to *Marboran*^R in inhibiting the multiplication of vaccinia virus in the mouse brain. Eight days after infection, when the virus titre in the control brains had already fallen to a low level, the differences between the control and treated groups were tending to disappear.

Considering that 40 μ mol of metisazone caused no death and 2.5 μ mol per mouse exerted at least 50% inhibition against 10^2 PFU of virus, the therapeutic index may be estimated at 16.

The potency test with the non-neurovirulent strain as based on the re-titration of the virus present in the mouse brain is too laborious for routine use.

Discussion

There is no generally accepted principle for the titration of the antiviral activity of drugs and the degree of protection achievable in man cannot be predicted on the basis of the results of the widely used methods of titration *in vivo* and *in vitro*. Owing to the lack of standard preparations, strains and methods, few of the results obtained in different laboratories are comparable.

The present investigations emphasize the necessity of standardizable methods for the titration of antiviral agents. We found the test *in ovo* superior to the tissue culture test for the titration of metisazone preparations. It should, however, be mentioned that we used chick embryo fibroblast cultures and presumably there are cell cultures more suitable for the purpose. Nevertheless, it does not seem justified to give preference to cell cultures against embryonated eggs in quantitative experiments with vaccinia virus.

Following up virus multiplication in the mouse brain provides some information about the effect of an antiviral drug *in vivo*, but this method is extremely laborious. To our best knowledge similar experiments with neurovirulent or non-neurovirulent strains have not been published.

The use of a neurovirulent vaccinia virus strain facilitates the titration of an antiviral drug *in vivo* as in this case re-titrations of the virus may be omitted and the absence of clinical or fatal illness may be accepted as an indicator of protection. In this respect the question arises whether a strain causing fatal illness is preferable to that causing only clinical symptoms.

It may be noted that we have repeated the present *in vivo* experiments with the neurovirulent strain IHD [28]. The therapeutic index calculated from this experiment was 40 as against the 8–16 calculated on the basis of experiments carried out with the Budapest strain.

At the beginning of the present study, we possessed no neurovirulent vaccinia virus strain. It was therefore attempted to select a neurovirulent variant from the Budapest strain, the strain used in Hungary for vaccine production, or to adapt this strain to the mouse brain, but these attempts failed. The Budapest strain had been derived from a sample of the Berlin strain received from DR. H. A. GINS in 1927. Since then the strain has been maintained in Budapest. Our unsuccessful adaptation and selection experiments seem to suggest that the Budapest strain, unlike the Berlin vaccine strain — which has been found neurovirulent and thus unsuitable for vaccine production by RÖHDE *et al.* [29] — is far from being neurovirulent for the mouse.

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EFFECT OF FLAVONOIDS AND RELATED SUBSTANCES

II. ANTIVIRAL EFFECT OF QUERCETIN, DIHYDROQUERCETIN AND DIHYDROFISSETIN*

By

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Summary. The effect on viruses of the flavonoids quercetin, dihydroquercetin and dihydrofisetin has been studied. They all displayed a virucidal effect on *Herpesvirus hominis* and *Herpesvirus suis*. Quercetin and dihydroquercetin inactivated herpesviruses at about the same rate. Parainfluenza virus type 3 was sensitive only to quercetin, while poliovirus type 2 was resistant to all the three substances.

Quercetin and dihydroquercetin were not only virucidal, but when given after infection they exhibited a virostatic effect by reducing the virus yield of *Herpesvirus hominis*.

The antiviral effect of the aqueous extract of dry oak leaves and of the aqueous solution of commercial tannic acid has been observed in this laboratory [1, 2]. Furthermore, it was shown that the antiviral activity of quercetin was similar to that of tannic acid [1, 3]. The present communication reports on comparative studies on the antiviral effect of certain flavonoids.

Materials and methods

Tissue culture and media. Tube cultures were seeded with 10^5 HeLa cells maintained in Gey's solution containing 5% rabbit serum and 0.25% lactalbumin hydrolysate.

For plaque assay 11-day chick embryos were trypsinized and 2.5×10^7 cells were seeded in Petri dishes 60 mm in diameter. The medium consisted of Gey's solution enriched with 5% calf serum, 0.25% lactalbumin hydrolysate, and 4% pH 7.6 Tris buffer. Sera were inactivated at 56°C for 30 minutes prior to use.

Virus assay. *Herpesvirus hominis*, poliovirus type 2, and parainfluenza virus type 3 were grown and titrated in HeLa cell cultures. The infectivity of *Herpesvirus suis* was determined by the plaque method on chick embryo fibroblast monolayers [2].

Preparation of flavonoid solutions. Stock solutions of 300 µg/ml concentration were prepared from quercetin (Merck), dihydroquercetin (Fluka, Buchs) and dihydrofisetin (Fluka, Buchs) in saline at a slightly alkaline pH adjusted by 0.2 N NaOH. Stock solutions were diluted as required in Hanks' solution.

Examination of antiviral effect

Assay of virucidal effect. To aliquots of the virus containing $10^{3.5}$ — $10^{4.5}$ TCID₅₀, the same volume of the flavonoid to be tested was added and the mixture was incubated at 20—25°C for 2 hours. Undiluted and tenfold diluted samples were inoculated to HeLa cell cultures. As controls, mixtures of virus and saline were used.

* Part of this paper was read at the II. National Bioflavonoid Symposium, Szeged, 1967.

In experiments with *Herpesvirus suis* 10^3 PFU/ml was mixed with equal volumes of flavonoid solution or saline. The mixture was titrated after appropriate incubation on chick embryo fibroblast monolayers.

Assay of virostatic effect. Each tube culture of HeLa cells was inoculated with 100 TCID₅₀ of *Herpesvirus hominis*. The cultures were incubated for 2 hours at 35°C. After removal of the inoculum the cultures were washed three times with Hanks' solution and 1 ml medium containing 100 µg quercetin or dihydroquercetin was added. For both substances tested 12 parallel tube cultures were used. The cultures were incubated at 35°C and the extra- and intracellular virus yields were determined after 24 and 72 hours. Extracellular virus was titrated in pooled supernatants centrifuged at 2500 r. p. m. for 15 minutes. To measure intracellular virus yield, the cultures were washed 3 times and the cells were suspended in 1 ml freshly added medium. The suspension thus obtained was pooled and disintegrated by sonication (M. S. E. ultrasonic disintegrator, 60 W) for 2 minutes. Cultures treated in the same way with saline instead of test material served as controls.

Results

The effect of 100 µg/ml solutions of quercetin, dihydroquercetin and dihydrofisetin was studied on *Herpesvirus hominis*, poliovirus type 2, and parainfluenza virus type 3. Results are shown in Table I. The degree of inactivation by flavonoids is expressed as the difference in the infective titre of treated and control virus samples.

Table I
Virucidal effect of flavonoids on different viruses

Flavonoid	Degree of inactivation (log)		
	<i>Herpesvirus hominis</i>	type 3 parainfluenzavirus	type 2 poliovirus
Quercetin	3.74	2.62	0.50
Dihydroquercetin	2.24	0.00	0.00
Dihydrofisetin	1.24	0.00	0.24

Table II
*Virostatic effect of quercetin and dihydroquercetin on *Herpesvirus hominis**

Flavonoid	Incubation (hours)	Virus yield log TCID ₅₀ /0.1 ml				Inhibition (log)	
		extracellular		intracellular		extra-cellular	intra-cellular
		control	treated	control	treated		
Quercetin	24	3.5	2.5	4.5	3.5	1	1
	72	5.74	4.5	5.5	4.5	1.24	1
Dihydroquercetin	24	3.5	1.5	4.5	3.74	2	0.76
	72	5.74	3.74	5.5	4.24	2	1.26

Quercetin was found to be the most effective of the three substances, being active against both herpes- and parainfluenza virus. As compared to the other two flavonoids, quercetin produced the greatest titre reduction of the infectivity of herpesvirus. The effectiveness decreased in the sequence, quercetin, dihydroquercetin, dihydrofisetin.

The virucidal effect of the flavonoids was examined on *Herpesvirus suis*. Results are shown in Fig. 1. Plaque counts were expressed as per cent of that of the control.

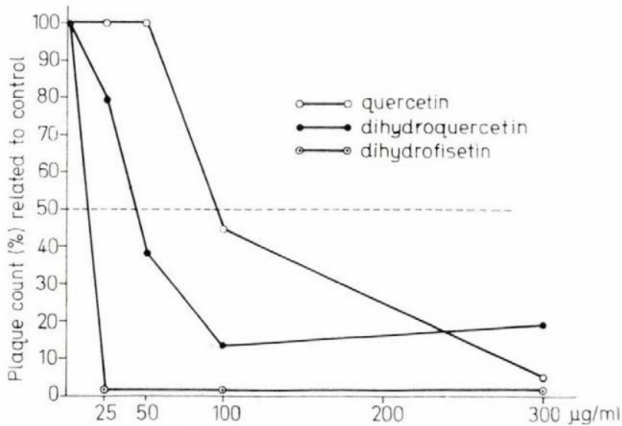


Fig. 1. Effect of flavonoids at different concentrations on *Herpesvirus suis*

The infectivity of the virus was completely destroyed by 25 $\mu\text{g/ml}$ of quercetin. Dihydroquercetin and dihydrofisetin at a concentration of 50 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ respectively caused a 50% reduction in plaque counts.

Of the viruses examined, herpesviruses were found to be the most sensitive to the virucidal effect of flavonoids. Therefore, to detect a possible virostatic effect, additional studies were performed on *Herpesvirus hominis*. Results are shown in Table II.

The substances reduced both the extra- and the intracellular virus yield. On extracellular virus, dihydroquercetin was the most effective. Intracellular virus yield was less affected by both flavonoids.

The eventual toxic effect of quercetin on HeLa cells was tested by incubating 10 tube cultures containing 100 μg quercetin each and 10 controls. On microscopic examination after 2 days incubation no morphological difference was detected between treated and control cells. Cell counts were determined after trypsinization of the cultures; the cell count was comparable in quercetin-treated and control cultures.

Discussion

The search for antiviral substances has recently been extended to a wide variety of plant extracts [4, 5, 6, 7, 8, 9]. In the course of these studies, tannic acid has been shown to have antiviral effect on certain viruses [2, 10, 11, 12, 13, 14]. The action of certain flavonoids on viruses has been examined in animal experiments by CUTTING *et al.* [15, 16, 17]. A prophylactic effect of quercetin and quercitrin against infection with rabies, neurovaccinia and ectromelia viruses has been demonstrated, nevertheless no effect was obtained when treatment has started after viral infection [15, 16, 17].

In our earlier studies aqueous extracts of dry oak leaves, tannic acid and quercetin were shown to have a virucidal effect on *Herpesvirus hominis*, *Herpesvirus suis* and on parainfluenza virus type 3 [1, 2, 3]. As rutin (quercetin-3-rutinoside) was found to be inactive, it seemed to be of interest to examine some additional flavonoids resembling quercetin. It was found that dihydroquercetin and dihydrofisetin exhibited an antiviral effect against herpesviruses. The antiviral effect of dihydroquercetin and quercetin was closely similar and surpassed that of dihydrofisetin. Parainfluenza virus type 3 was sensitive only to quercetin and poliovirus type 2 appeared resistant to all the three flavonoids.

Quercetin and dihydroquercetin were shown to have not only a virucidal, but also a virostatic effect against *Herpesvirus hominis*. The extracellular virus yield was most markedly reduced by dihydroquercetin, though this effect was weaker than in the experiment concerning virucidal effects. The difference was probably due to certain disparities in the experimental conditions.

In contrast to the other flavonoids, rutin failed to have any effect on herpesviruses. It is therefore supposed that the antiviral activity of flavonoids requires the presence of a hydroxyl residue on the carbon atom in position 3. The double bond between the carbon atoms in positions 2 and 3 did not seem to be important, as quercetin and dihydroquercetin were equally active. Further studies are needed to prove this assumption.

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THE EFFECT OF HYPOTHERMIA ON THROMBOHAEMORRHAGIC REACTIONS

By

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Summary. Endotoxin was injected into the pancreas and salivary gland of rabbits then the Schwartzman reaction was elicited. If the intravenous eliciting dose was given to animals under hypothermia, the reaction was slight or absent. The Schwartzman reaction induced with leukocyte homogenate could also be inhibited by cooling the animals. In contrast, after treatment with ScCl_3 + adrenaline, histamine, 48/80, or 5-HT as preparatory and NaCl as eliciting agent, rats developed a necrotic dermal reaction independently of their body temperature.

It has been shown that hypothermia effectively inhibited or eliminated the Schwartzman reaction [1]. The protective effect was observed only when the animals had been cooled prior to the administration of the eliciting dose. In 1961 BURACK and MOSIMANN [2] reported that the local Schwartzman phenomenon is a temperature dependent reaction. The present paper gives an account of the effect of hypothermia on the Schwartzman reaction in organs and on other thrombohaemorrhagic reactions.

Materials and methods

Local Schwartzman phenomenon was induced in the pancreas and salivary gland of rabbits. After hexobarbital anaesthesia the pancreas and the superficial mandibular gland were exposed under aseptic conditions and the preparatory endotoxin dose (*E. coli* 0111) was injected. Provocation was performed on the next day by giving endotoxin intravenously to cooled and control animals anaesthetized with barbiturate. The region of the salivary gland was cooled also locally with an ice bag.

Four to 6 hours after the eliciting injection specimens of the pancreas and salivary gland were excised for histological examination. After fixation in Susa's solution and formalin and embedding in paraffin, the sections were stained with haematoxylin and eosin.

Leukocyte homogenate was prepared by a method adapted from COHN and HIRSCH [3]. The stock suspension of 10^7 leukocytes per ml was diluted 1 : 2 and 1 : 3 then injected intracutaneously at 0.4 ml doses into the shaven dorsal skin of the animals. Local Schwartzman reaction was elicited after 16 hours with the intravenous injection of endotoxin.

Thrombohaemorrhagic reaction [4] and acute conditioned necrosis [5] were performed in rats as follows. Each animal was given 3 mg ScCl_3 intravenously and 200 μg adrenaline into the paw. Histamine was administered intravenously in 30 mg, 48/80 in 0.1 mg, and 5-HT in 1 mg doses. The eliciting dose for animals receiving these substances consisted of injecting under the dorsal skin 200 mg NaCl in 2 ml of distilled water. The rats were anaesthetized with barbiturate and cooled in an ice bath. The rectal temperature, recorded by a thermocouple, was in the range of 21 to 25°C.

Results

In the 6 normothermic control animals the pancreas showed definite and characteristic changes consisting of parenchymal round cell infiltration, thrombosis affecting the majority of small vessels, faint nuclear staining and beginning necrosis (Fig. 1). In the 6 hypothermic animals the pancreas was characterized by interstitial round cell infiltration, the nuclei showed normal staining and thrombosis was absent (Fig. 2). This picture corresponded to the changes appearing after local administration of the endotoxin without a subsequent eliciting injection.

The local Shwartzman reaction in the salivary gland was characterized by extensive necrosis in all the 6 normothermic animals (Fig. 3). On general and local cooling (6 animals) there was solely an interstitial round cell infiltration characteristic of the effect exerted by the preparatory endotoxin dose *per se* (Fig. 4).

Table I

Local Shwartzman reaction in normothermic rabbits

Animal No.	Dilution of leukocyte homogenate	Rectal temperature at provocation, °C	Size of reaction
1	1 : 3	37.2	30/24
2	1 : 3	36.9	25/23
3	1 : 3	37.0	20/20
4	1 : 3	37.1	18/18
5	1 : 3	36.8	24/20
6	1 : 3	36.9	25/23
7	1 : 3	37.1	29/27
8	1 : 3	36.8	17/14
9	1 : 3	37.0	24/19
10	1 : 3	37.0	16/14
11	1 : 2	36.9	41/25
12	1 : 2	37.3	50/23
13	1 : 2	37.0	70/48
14	1 : 2	37.0	50/50
15	1 : 2	36.9	34/32
16	1 : 2	37.1	30/30

Preparatory dose: leukocyte homogenate; eliciting dose: intravenous endotoxin. Size of reaction: largest/smallest diameter of haemorrhagic area in mm.

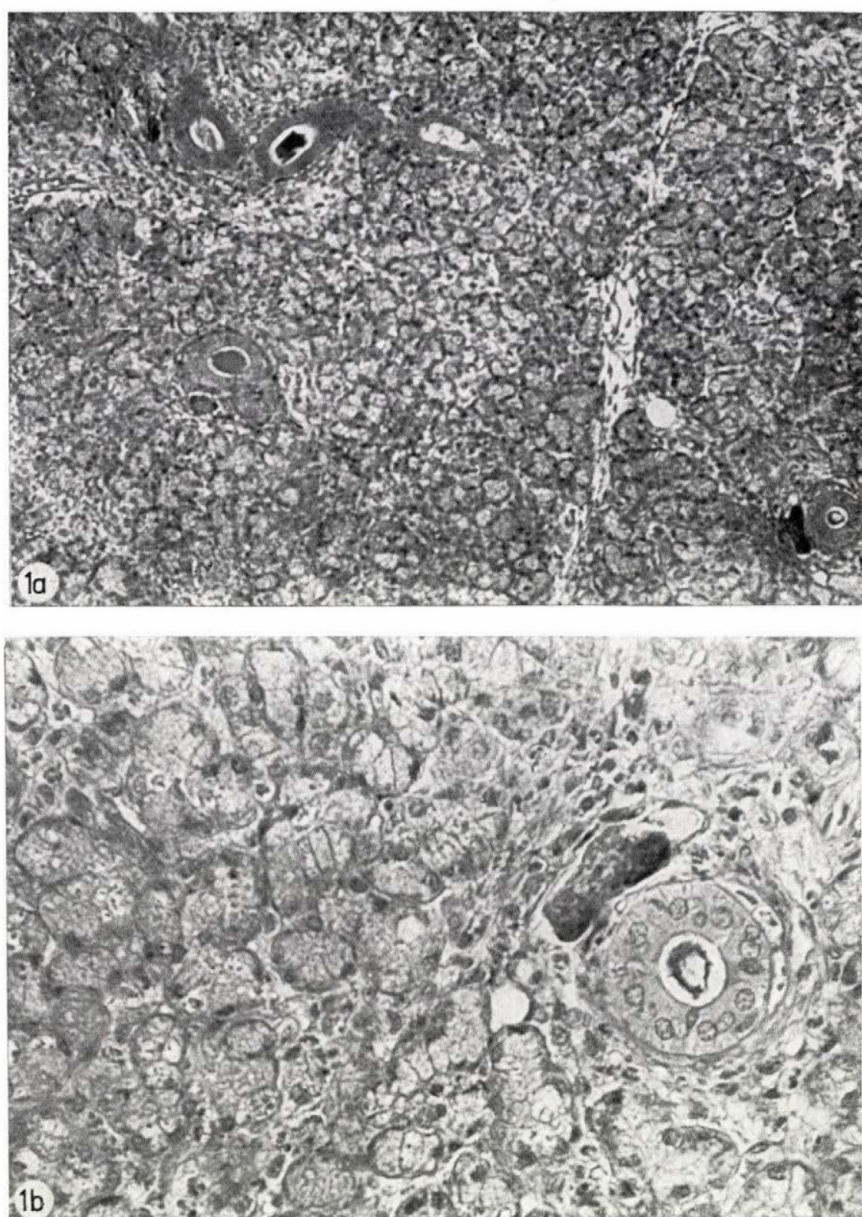


Fig. 1. Reaction in the pancreas of normothermic animals. Round cell infiltration, obliteration of small vessels, perivascular oedema, faint nuclear staining. Haematoxylin-eosin, a: $\times 150$, b: $\times 480$

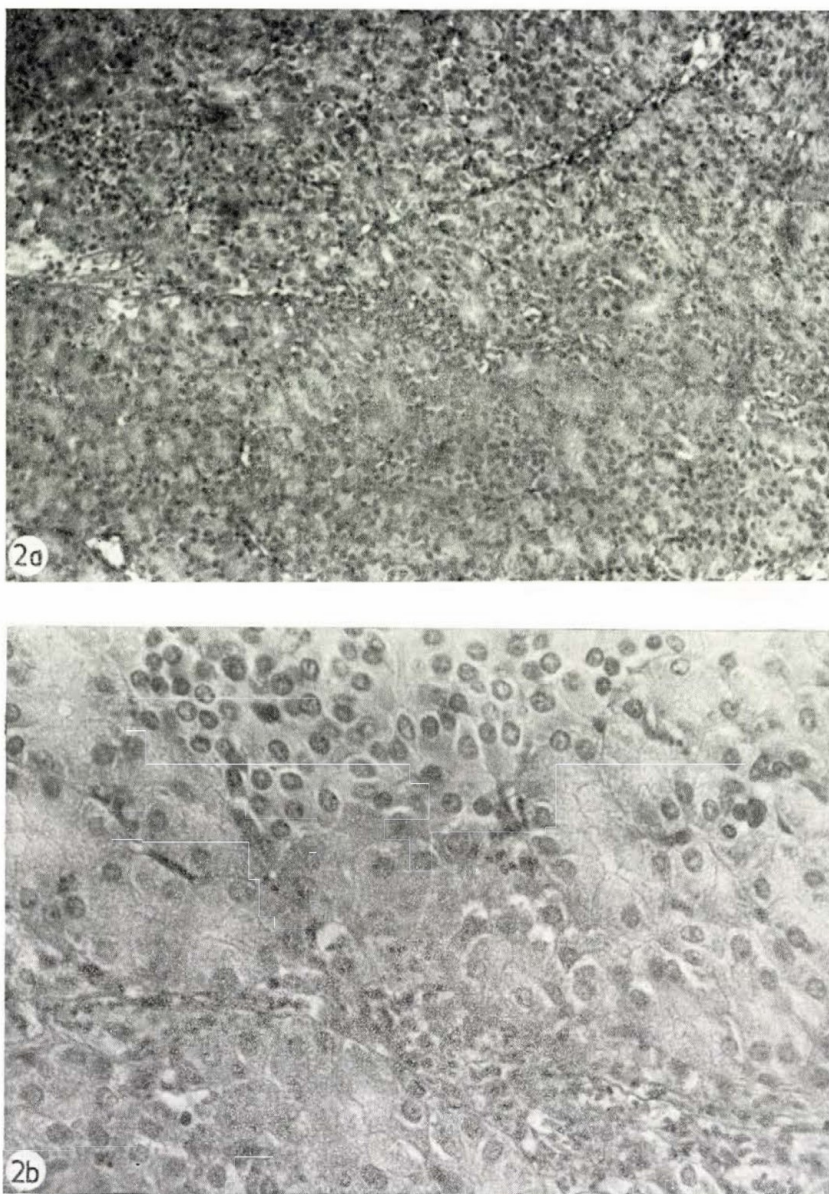


Fig. 2. Reaction in the pancreas of hypothermic animals. Round cell infiltration, normal nuclear staining; no obliterated vessels. Haematoxylin-eosin, a: $\times 150$, b: $\times 480$

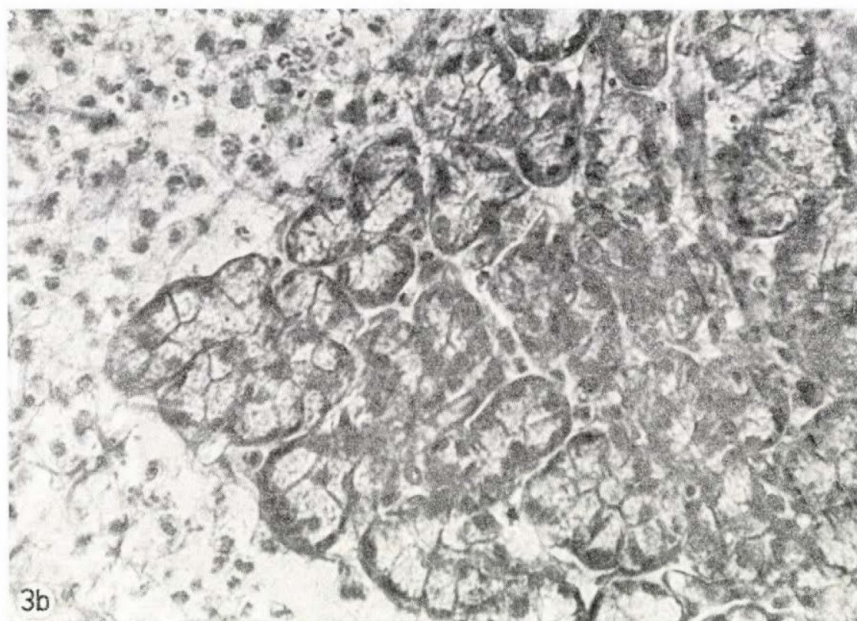


Fig. 3. Reaction in the superficial mandibular gland of normothermic animals. Advanced haemorrhagic necrosis (a); karyopyknosis, faint nuclear staining, necrosis (b). Haematoxylin-eosin, a: $\times 150$, b: $\times 480$

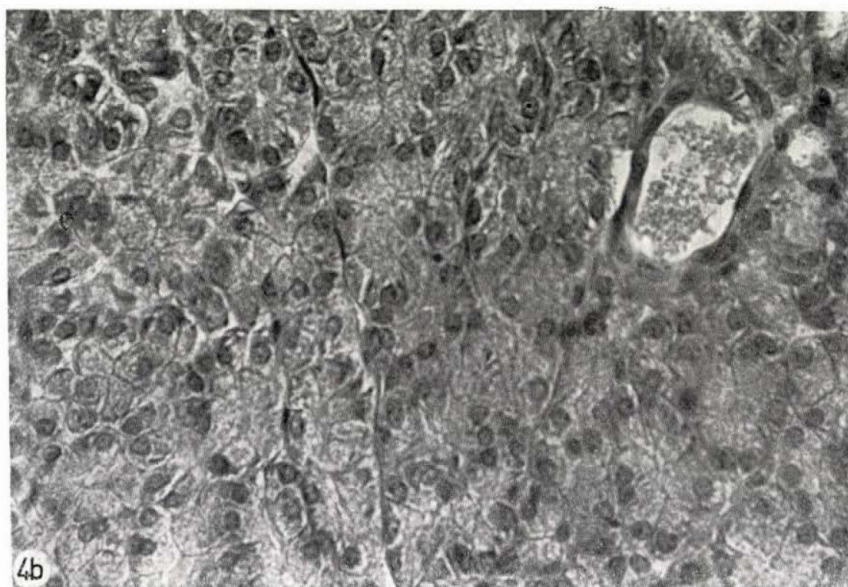
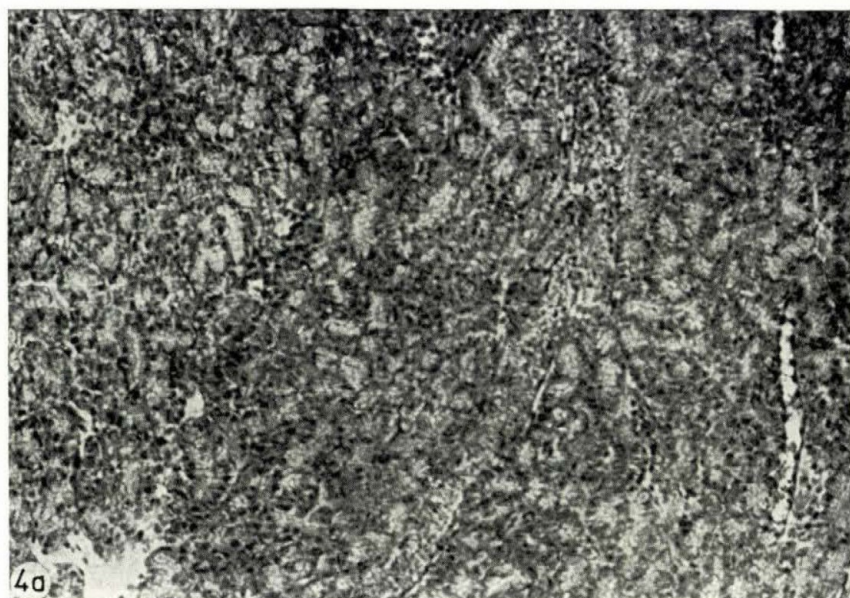


Fig. 4. Reaction in the superficial mandibular gland of hypothermic animals. Interstitial round cell infiltration, normal nuclear staining; no obliterated vessels. Haematoxylin-eosin, a: $\times 150$, b: $\times 480$

Experiments with the leukocyte homogenate were performed as described by THOMAS [6]. A definite local Shwartzman reaction developed in normothermic animals receiving the leukocyte homogenate diluted 1 : 3. The reaction was severe in rabbits which had been given the preparation diluted 1 : 2 (Table I).

Hypothermic rabbits receiving the homogenate in 1 : 3 dilution were protected against the local reaction. In animals injected with the homogenate diluted 1 : 2 the haemorrhagic reaction was smaller than in the control rabbits, but was not completely prevented.

Examinations as to whether hypothermia inhibited the thrombohaemorrhagic phenomenon and acute conditioned necrosis were performed in groups of rats each consisting of 10 animals. It was shown that these reactions developed at identical intensity in hypothermic and in normothermic animals (Fig. 5).

Table II

Local Shwartzman reaction in hypothermic rabbits

Animal No.	Dilution of leukocyte homogenate	Rectal temperature at provocation, °C	Size of reaction
17	1 : 3	27.5	—
18	1 : 3	28.0	6/4
19	1 : 3	26.5	—
20	1 : 3	27.0	—
21	1 : 3	27.0	—
22	1 : 3	26.8	—
23	1 : 3	27.0	—
24	1 : 3	28.0	8/8
25	1 : 3	27.8	—
26	1 : 3	28.2	12/12
27	1 : 2	27.0	16/12
28	1 : 2	26.8	18/18
29	1 : 2	27.0	14/12
30	1 : 2	27.2	22/20
31	1 : 2	27.0	24/24
32	1 : 2	26.8	20/16

Preparatory dose: leukocyte homogenate; eliciting dose: intravenous endotoxin. Size of reaction: largest/smallest diameter of haemorrhagic area in mm.

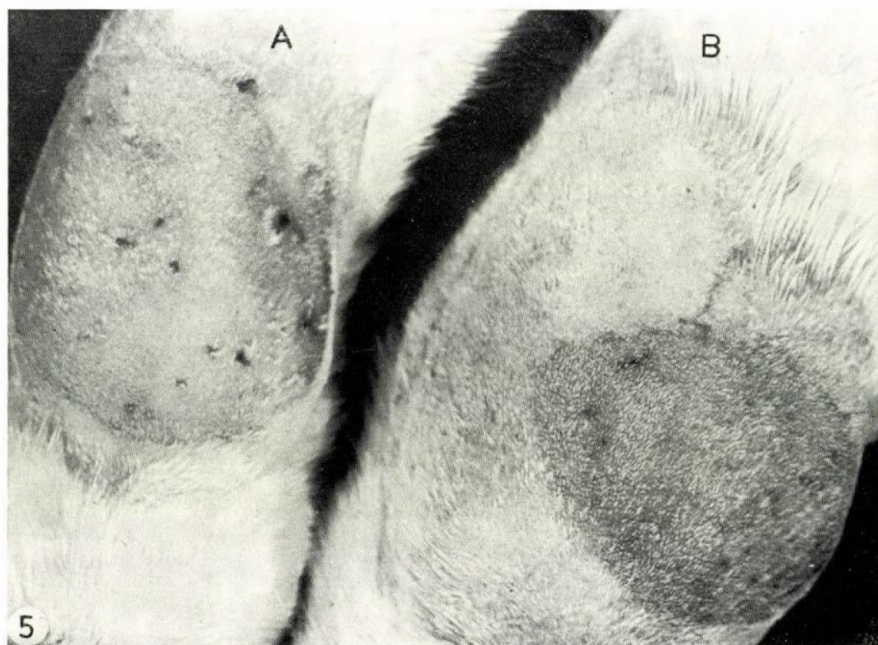


Fig. 5. Acute conditioned necrosis in rats. Preparatory injection: 5-HT intravenously; eliciting injection: NaCl subcutaneously. A: normothermic animal, B: hypothermic animal

Discussion

Several authors have demonstrated that local Schwartzman reaction can be produced in the pancreas [7–9]. Recently it has been described that experimental pancreatitis is inhibited by hypothermia; the lesion was, however, induced with agents other than endotoxin [10, 11].

Our results indicate that hypothermia highly inhibits the local Schwartzman reaction in the pancreas if the eliciting injection is given to animals which have been cooled to low body temperatures. The finding may partly be explained by the fact that in hypothermia both the secretion and the activity of pancreatic enzymes is considerably decreased [12].

In the available literature no data have been found as to the local Schwartzman reaction in salivary glands. In our experiments hypothermia inhibited the Schwartzman reaction in these glands more definitely than in the pancreas; difference was probably due to the effectiveness of the local cooling of the salivary gland.

Experiments with leukocyte homogenate showed that the intensity of the Schwartzman reaction is determined by the amount of the preparatory

substance. If the usual doses were given, hypothermia inhibited the development of the reaction similarly as it prevents the endotoxin-induced reaction. The reaction to leukocyte homogenate doses causing extremely severe lesions in the control animals was, however, not prevented but only decreased by cooling.

The present experiments confirm our earlier finding that the local Shwartzman reaction depends on body temperature. This consideration holds not only for skin reactions, but also for reactions elicited in secretory glands and for the Shwartzman phenomenon induced by leukocyte homogenate. Our results and the findings of BURACK and MOSIMANN [2] uniformly indicate that the processes occurring after the administration of the eliciting dose are influenced by body temperature. The hypothermic condition does not inhibit the reaction if the animals are cooled at the time of the preparatory injection or after the administration of the eliciting dose. In the opinion of UTSHITEL and KRIMSKY [13], inhibition of the local Shwartzman reaction is associated with the mobilization of corticosteroids in the hypothermic condition. We have shown that large doses of cortisone exerted some inhibitory action on this process [14.] In view of this finding and the observation that cooling of anaesthetized animals causes a very slight corticosteroid mobilization [15, 16], the hypothesis advanced by UTSHITEL and KRIMSKY should be rejected. The process involved in the inhibition of the Shwartzman reaction in hypothermia should be elucidated in further experiments.

Our results clearly indicate that hypothermia exerts no protective effect against either the thrombohaemorrhagic reaction induced by ScCl_3 + adrenaline or the acute conditioned necrosis produced by various agents. This finding suggests that, although these phenomena and the Shwartzman reaction are similar in appearance, two different mechanisms are involved.

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CONVERSION OF STEROIDS BY *ALCALIGENES* *FAECALIS*

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Summary. One of our bile acid-decomposing isolates has been identified as *Alcaligenes faecalis*. The organism exerts Δ^1 and Δ^4 -dehydrogenase, 3β -hydroxy-dehydrogenase, Δ^5 , Δ^4 -isomerase and esterase activity and cleaves the steroid nucleus between C-9 and C-10. The rate of metabolism of various androstane and pregnane compounds has been compared.

By use of an enrichment method we have isolated bile acid-decomposing microorganisms from soil [1]. Among the isolates using lithocholic acid as a sole source of carbon, strain K 9—2 was extensively investigated for steroid-converting capacity. The most important reactions in the microbiological conversion of steroids are the production, isomerization and reduction of carbon-carbon double bonds, incorporation and oxidation of hydroxyl groups and cleavage of carbon-carbon and ester bonds. The microorganism has been identified and characterized by its ability to convert various steroids.

Materials and methods

Cultivation and microbiological conversion. *A. faecalis* strain K 9—2 was maintained on potato-glucose agar. Two-day agar slant cultures grown at 28°C were suspended in sterile distilled water. One ml of the suspension was used for the inoculation of 100 ml SA_{5-g} medium.

SA_{5-g} medium contained peptone, 50 g; meat extract, 10 g; brewer's yeast extract, 2 ml; the volume was made up to 1000 ml with tap water and the pH was adjusted to 6.5—7.0. The medium was distributed at 100 ml portions into 500 ml Erlenmeyer flasks and autoclaved at 121°C for 15 minutes.

The seeded flasks were shaken in a 28°C incubator room for 16 hours on a rotary shaker describing a 30 mm circle at 180 r.p.m. Then each 100 ml culture was supplemented with 20 mg of steroid dissolved in 1 ml acetone.

The flasks were shaken further at 28°C and sampled at two hour intervals for the determination of steroid composition.

Steroid conversion. Qualitative examination of the metabolism of steroid substrates was performed by thin layer chromatography on Silicagel G (Merck). The chromatograms were run from the ethylacetate extracts of the fermentation liquors. The solvents used for the development were chloroform containing 2% ethanol for 17-ketosteroids; chloroform containing 3% ethanol for 17 α -methyl and 17 β -hydroxy-steroids; chloroform containing 4% ethanol for corticoids unsubstituted at C-11; dioxane-benzene mixture (4 : 6) for corticoids with an oxygen functional group at C-11. The spots were detected at 100°C for 10 minutes after spraying with a 1 : 1 mixture of ethanol and sulphuric acid.

*Deceased in 1965

Determination of $\Delta^{1,4}$ -3-ketosteroids in the fermentation liquor was performed by utilizing data for the ultraviolet absorption maxima of ketosteroid thiosemicarbazones saturated to different degrees in the steroid A ring [2-4]. The thiosemicarbazones of saturated A ring Δ^4 and $\Delta^{1,4}$ -3-ketosteroids exhibited the following ultraviolet absorption maxima: $\lambda_{\max}$ saturated 3-ketone: 270-275 nm; $\lambda_{\max}$ Δ^4 -3-ketone: 301-302 nm; $\lambda_{\max}$ $\Delta^{1,4}$ -3-ketone: 323-325 nm. Thiosemicarbazones produced from saturated steroid-ketones did not show absorption at the maximum for $\Delta^{1,4}$ -3-ketosteroid-thiosemicarbazones and thus did not interfere with estimation. The absorption of Δ^4 -3-ketosteroid thiosemicarbazones which coincides with the absorption maximum for $\Delta^{1,4}$ -3-ketosteroid thiosemicarbazones, could be eliminated by the method used for the spectrophotometric determination of two-component systems.

The determinations were performed as follows. Four ml volumes of the fermentation liquor were extracted in a separation funnel with 15 ml water-immiscible solvent (carbon tetrachloride, ethylene chloride or ethyl acetate, depending on the polarity of the steroid). The organic phase was dehydrated with Na_2SO_4 , then a 1 ml portion was dried by evaporation in a glass-stoppered test tube. After dissolving the residue in 1 ml of 96% ethanol, 2 ml 0.5 M thiosemicarbazide dissolved in 0.5 N HCl was added and the test tube was incubated at 40°C for 30 minutes. Then 10 ml of 96% ethanol was added and the optical densities were read at the absorption maxima for Δ^4 -3-ketosteroid and $\Delta^{1,4}$ -3-ketosteroid thiosemicarbazones ($\lambda = 300-302$ and $\lambda = 323-325$, respectively). A similarly treated solution was used as the blank. $\Delta^{1,4}$ -3-ketosteroid content (c) was calculated by use of the formula

$$c = k_1 \cdot e_{323-325} - k_2 \cdot e_{300-302}$$

where $e_{323-325}$ and $e_{300-302}$ are the optical densities measured at the absorption maxima for $\Delta^{1,4}$ -3-ketosteroid thiosemicarbazones and Δ^4 -3-ketosteroid thiosemicarbazones, respectively, and k_1 and k_2 the constants characteristic of the two compounds.

Isolation of steroids produced during metabolization. Isolation of 17 α -methyl-androsta-1,4-diene-17 β -ol-3-one. In 50 shaken flasks 17 α -methyl-androst-4-ene-17 β -ol-3-one was fermented for 16 hours as described under "Cultivation and microbiological conversion". The combined fermentation liquors were extracted three times with 1/6 volumes of carbon tetrachloride. The extract was evaporated under reduced pressure. The residue was crystallized from ether. The crude preparation was then recrystallized twice from ethyl acetate: m.p. 164-167°C, $[\alpha]_D = +7^\circ$ (c = 95% ethanol), $\lambda_{\max} = 245$ nm, $E_{1\text{cm}}^{1\%} = 516$, identical in infrared spectrum with the authentic specimen.

Isolation of androsta-1,4-diene-3,17-dione. Fermentation of androst-4-ene-3,17-dione and carbon tetrachloride extraction were performed as described above. After evaporation *in vacuo* the residue was crystallized from ether: m.p. 139-142°C, $[\alpha]_D = +115^\circ$ (c = 1, chloroform), $\lambda_{\max} = 244$ nm, $E_{1\text{cm}}^{1\%} = 538$, identical in infrared spectrum with the authentic specimen.

Isolation of pregna-1,4-diene-17 α , 21-diol-3,20-dione. Fermentation of pregn-4-ene-17 α , 21-diol-3,20-dione was carried out as described above. Extraction was made three times with 1/6 volumes of 1,2-dichloroethane. After evaporating to dryness the residue was treated with hexane. The hexane-insoluble crystalline crude preparation was recrystallized from acetone: m.p. 234-236°C, $[\alpha]_D = +74^\circ$ (c = 1, acetone), $\lambda_{\max} = 244$ nm, $E_{1\text{cm}}^{1\%} = 445$, identical in infrared spectrum with the authentic specimen.

Isolation of 9,10-seco-androsta-1,3,5(10)-triene-3-ol-9,17-dione. Fermentation was carried out in 100 flasks for 24 hours in the above described manner. The combined fermentation liquor was extracted three times with 1/3 volumes of 1,2-dichloroethane. The extract was evaporated to 100 ml volume under reduced pressure. The concentrate was washed twice with 100 ml volumes of 5% NaHCO_3 in order to remove products containing carboxylic group. Extraction was then performed three times with 50 ml volumes of 1,2-dichloroethane. The extract was evaporated to dryness and crystallized from mixture of ether and petroleum ether. The crude preparation was recrystallized from an acetone-petroleum ether mixture: m.p. 122-124°C, $[\alpha]_D = +97^\circ$ (c = 1, chloroform), $\lambda_{\max} = 280$ nm, $E_{1\text{cm}}^{1\%} = 78$, identical in infrared spectrum with the authentic specimen.

Results

Morphological properties and determination of the organism. Rodshaped cells with rounded ends $2-2.5 \times 0.8 \mu$ in size. The rods are frequently arranged in pairs. Motile: electrone micrographs revealed at each pole several slightly wavy flagella longer than the bacterial cell. Phase contrast microscopy

showed the presence of bipolar, more or less refractive granules. Gram negative.

The organism corresponded in main morphological and biochemical properties to *Alcaligenes faecalis* described in *Bergey's Manual* [5]. The following differences were noted. According to BERGEY, *A. faecalis* has a temperature optimum of 37°C, is peritrichous and produces no characteristic odour. Our strain grew better at 24 to 28°C than at 37°C, was lophotrichous and smelled of

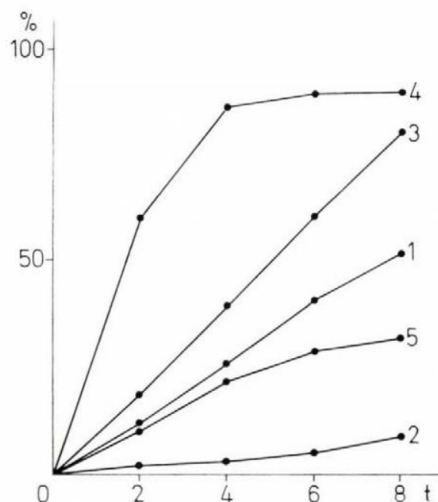


Fig. 1. Conversion of androstane series 17 α -methyl-17 β -hydroxy steroids. Abscissa: fermentation time in hours; ordinate: 17 α -methyl-androsta-1,4-diene-17 β -ol-3-one as per cent of substrate. Substrates: 1 = 17 α -methyl-5 α -androstane-3 β -17 β -diol; 2 = 17 α -methyl-androsta-5-ene-3 β ,17 β -diol; 3 = 17 α -methyl-5 α -androstane-17 β -ol-3-one; 4 = 17 α -methyl-androst-4-ene, 17 β -ol-3-one; 5 = 17 α -methyl-androst-5-ene-17 β -ol-3-one

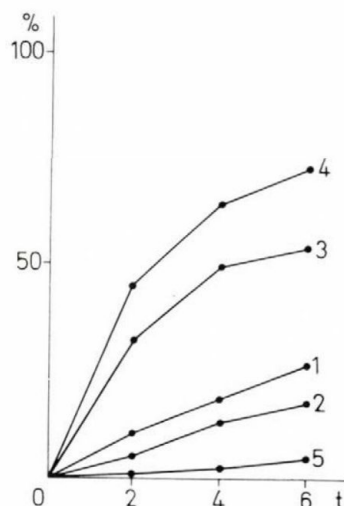


Fig. 2. Conversion of androstane series 17-ketosteroids. Abscissa: fermentation time in hours; ordinate: androsta-1,4-diene-3,17 dione as per cent of substrate. Substrates: 1 = 5 α -androstane-3 β -ol-17-one; 2 = androst-5-ene-3 β -ol-17-one; 3 = 5 α -androstane-3,17-dione; 4 = androst-4-ene-3,17-dione; 5 = androst-5-ene-3 β -ol-17-one-3 β -acetate

fish. SKERMAN [6] states that strains with a low temperature optimum and possessing polar flagella may belong to *A. faecalis*, but does not mention the smell. On the basis of these findings we identified our strain as *A. faecalis*.

Metabolization of androstane series 17 α -methyl-17 β -hydroxy steroids. Fig. 1 indicates that 5 α -saturated 3 β -hydroxy, Δ^5 -3 β -hydroxy, 5 α -saturated 3-keto, Δ^4 -3-keto, Δ^5 -3-keto compounds are converted at various rates to the $\Delta^{1,4}$ -3-keto compound 17 α -methyl-androsta-1,4-diene, 17 β -ol-3-one.

The qualitative thin layer chromatogram was in good agreement with spectrophotometric estimations. The degree of the conversion of 17 α -methyl-androsta-5-ene-3 β ,17 β -diol was slight in 8 hours; during 48 hours incubation, however, a well-defined amount of 17 α -methyl-androsta-1,4-diene-3,17-dione had formed through the appropriate intermediary products.

Metabolization of androstane series 17-ketosteroids. Fig. 2 indicates that the substrates examined were converted to the $\Delta^{1,4}$ -3-keto compound androsta-1,4-diene-3,17-dione.

After 8 hours fermentation chromatography showed but traces of androsta-1,4-diene-3,17-dione in the androst-5-ene-3 β -ol-17-one-3 β -acetate fermentation. The first step of androst-5-ene-3 β -ol-17-one-3 β -acetate metabolization, hydrolysis of the ester group at position 3 took as long as 24 hours.

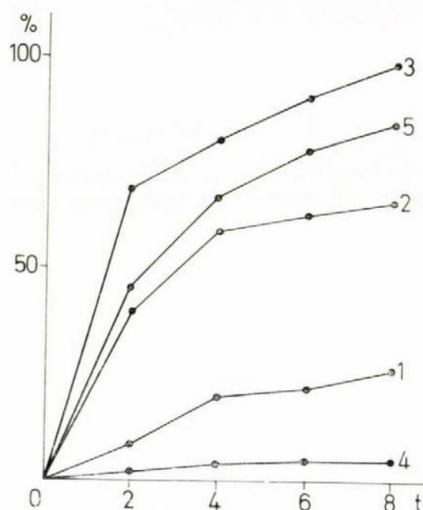


Fig. 3. Conversion of corticosteroids. Abscissa: fermentation time in hours; ordinate: pregn-1,4-diene-17 α ,21-diol-3,20-dione as per cent of substrate. Substrates: 1 = pregn-5-ene-3 β ,17 α ,21-triol-20-one; 2 = 5 α -pregnane-17 α ,21-diol-3,20-dione; 3 = pregn-4-ene-17 α ,21-diol-3,20-dione; 4 = pregn-5-ene-3 β ,17 α ,21-triol-20-one-3 β ,21-diacetate; 5 = pregn-4-ene-17 α ,21-diol-3,20-dione-21-acetate

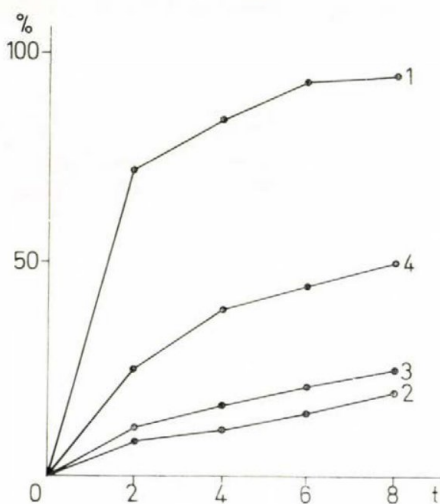


Fig. 4. Rate of Δ^1 -dehydrogenation for various corticoids. Abscissa: fermentation time in hours; ordinate: Δ^1 -dehydro product as per cent of substrate. Substrates: 1 = pregn-4-ene-17 α ,21-diol-3,20-dione; 2 = pregn-4-ene-11 α ,17 α ,21-triol-3,20-dione; 3 = pregn-4-ene-11 β ,17 α ,21-triol-3,20-dione; 4 = pregn-4-ene-17 α ,21-diol-3,11,20-trione

Metabolization of corticosteroids. Fig. 3 shows the conversion of corticoids unsubstituted at C-11 to pregn-1,4-diene-17 α ,21-diol-3,20-dione.

As shown in chromatographic experiments, all substrates yielded pregn-1,4-diene-17 α ,21-diol-3,20-dione. The metabolization of pregn-5-ene-3 β ,17 α ,21-triol-20-one and especially of pregn-5-ene-3 β ,17 α ,21-triol-20-one-3 β ,21-diacetate was slow and could only be studied by observing the fermentation for 72 hours. It was also revealed that the acetate group at position 21 of pregn-4-ene-17 α ,21-diol-3,20-dione-21-acetate was hydrolysed in approximately 8 hours.

Fig. 4 presents a comparison of the rate of Δ^1 -dehydrogenation for corticoids possessing an oxygen functional group at C-11 and for unsubstituted corticoids.

Results were in agreement with the chromatographic findings. All substrates had been converted to the corresponding dehydro product, but the Δ^1 -dehydrogenation rate varied widely with each substrate.

General observations on the steroid metabolism by A. faecalis. The micro-organism decomposed very slowly the $\Delta^{1,4}$ -3-keto compounds accumulating during conversion. Thus from the increase in the concentration of these substances the rate of substrate conversion could be estimated.

The slow breakdown of $\Delta^{1,4}$ -3-keto compounds was also studied. During the fermentation of androsta-1,4-diene-3,17-dione, parallel with the decrease in substrate concentration 9(10)-seco-androsta-1,3,5(10)-triene-3-ol-9,17-dione appeared in increasing amounts.

Thin layer chromatography revealed that the conversion of all substrates took place in the presence of substances inhibiting protein metabolism such as 100 $\mu\text{g/ml}$ of chloramphenicol. Chloramphenicol at 200 $\mu\text{g/ml}$ concentration, however, completely inhibited the conversion even when the culture had been preincubated for 2 to 6 hours in a medium containing 20 $\mu\text{g/ml}$ of the examined steroid.

Discussion

It has been concluded that our *A. faecalis* strain exerted Δ^1 and Δ^4 dehydrogenase, 3β -hydroxy-dehydrogenase, Δ^4 — Δ^5 isomerase and esterase activity, and accumulated $\Delta^{1,4}$ -3-keto compounds during metabolism.

From the comparison of the Δ^1 -dehydrogenation of androstane series compounds it was evident that methyltestosterone which belongs to the 17α -methyl- 17β -hydroxy compounds, was dehydrogenated somewhat more rapidly than androst-4-ene-3,17-dione which belongs to the 17-ketosteroid group.

The dehydrogenation of corticoids possessing an oxygen functional group at C-11 was slower than that of substrates unsubstituted at C-11 (preg-4-ene- 17α ,21-diol-3,20-dione); the difference was especially marked in the case of hydrocortisone and epihydrocortisone containing a hydroxyl group at C-11. These findings indicate that the rate of Δ^1 -dehydrogenation decreases with the increase in polarity.

As compared to Δ^1 and Δ^4 dehydrogenase activity, *A. faecalis* exerted a considerably weaker 3β -hydroxydehydrogenase and Δ^4 — Δ^5 isomerase activity.

As to esterase activity, it was observed that the organism splits the 21-acetyl group (primary hydroxyl ester) much more rapidly than the 3β -acetyl group (secondary hydroxyl ester).

The isolation of 9(10)-seco-androsta-1,3,5(10)-triene-3-ol-9,17-dione from androsta-1,4-diene-3,17-dione fermentation indicates that fission of the steroid ring occurs between C-9 and C-10 by a metabolic pathway observed with *Mycobacterium smegmatis* and *Nocardia restrictus* [7,8].

From experiments with substances inhibiting the metabolism of protein it has been concluded that constitutive enzymes are involved in the steroid conversions examined.

Earlier studies of SCHMIDT *et al.* [9, 10] showed that *A. faecalis* oxidizes the 3,7 and 12 hydroxyl groups of steroids into ketone. They used mainly bile acids as substrates, but also observed the oxidation of dehydroepiandrosterone to androst-4-ene-3,17-dione.

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INCIDENCE AND BIOLOGICAL PROPERTIES OF METHICILLIN AND OXACILLIN RESISTANT STAPHYLOCOCCUS AUREUS STRAINS

By

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Summary. The incidence of methicillin and oxacillin-resistant *Staph. aureus* strains is increasing. In clinical material, resistant cultures occurred in 4.3—4.6% in 1966, and in 5.7—7.9% in 1967. Tests in vitro considered to indicate pathogenicity failed to reveal any significant difference between resistant and sensitive staphylococci. Although resistant strains occurred in all phage groups, most of them belonged to phage group III or were untypable. Naturally occurring methicillin and oxacillin resistant cultures consisted of mixed populations; the incidence of resistant colony-producing units was 1/23000 to 1/79000. There was a characteristic difference in the type of multiplication in the presence of methicillin between naturally occurring and laboratory-induced methicillin-resistant strains.

In recent years methicillin and oxacillin have widely been used in therapy. Although the pharmacological, clinical and in vitro antibacterial properties of these antibiotics have extensively been investigated in Hungary [2, 3, 10, 13—15, 18], less attention has been paid to the incidence and biological properties of methicillin and oxacillin-resistant *Staphylococcus aureus* strains. This paper reports on the biological properties of resistant staphylococci isolated from patients treated in different departments of the University Medical School, Debrecen.

Materials and methods

Strains and antibiotic sensitivity. The total number of *Staph. aureus* strains isolated was 861 in 1966 and 617 in 1967. All strains were tested for antibiotic sensitivity with the disc method by seeding 6 hour broth cultures on sheep blood agar plates. The antibiotic content of methicillin and oxacillin discs was 20 µg and 10 µg, respectively. Each strain shown to be resistant by the disc method was examined by the two-fold serial dilution technique using 1 ml volumes of 0.5% glucose Casitone broth (Difco). Each tube was inoculated with 0.1 ml of a 1 : 100 dilution of a 24 hour culture prepared in the same medium, then incubated for 48 hours at 37°C. Strains inhibited by at least 16 µg per ml of methicillin or 8 µg per ml of oxacillin were considered resistant.

Properties of the strains. Pigment and haemolysin production was examined on sheep blood agar after incubation at 37°C for 24 hours and a subsequent standing at room temperature for another 24 hours. Coagulase activity was tested by the slide method. Acid production from mannitol and urease production were examined after 24 hours incubation in 1% mannitol peptone water and in SZITA's medium, respectively. Liquefaction of gelatin was tested in 10% gelatin medium incubated for 10 days at room temperature.

Phage typing was performed as described by WILLIAMS and RIPPON [19].

Population analysis was carried out on blood agar plates containing methicillin and oxacillin at various concentrations. Twenty-four hour 0.5% glucose Casitone broth cultures were diluted 10^{-2} , 10^{-4} , 10^{-6} and 10^{-7} with saline then 0.1 ml aliquots of each dilution were plated. The 10^{-6} and 10^{-7} dilutions were plated also onto antibiotic-free blood agar in order to determine the number of colony-producing units. The plates were incubated at 37°C for 48 hours.

Growth curves for cultivation in the presence of methicillin and oxacillin were determined at 37°C in a Bonet Maury et Jouan biophotometer, for 48 hours.

Results

Table I shows the incidence of methicillin and oxacillin-resistant strains in 1966 and 1967. Resistant staphylococci occurred in both years most frequently in infections of the ear and the respiratory tract. The incidence of resistant strains in these materials increased in 1967 as compared to the previous year. The rising tendency was especially evident in other conditions such as biliary, superficial eye, and urogenital infections.

Table I

Incidence of methicillin and oxacillin-resistant Staph. aureus strains in the years 1966 and 1967

Source	Year	No. of strains	Methicillin-resistant strains		Oxacillin-resistant strains	
			No.	per cent	No.	per cent
Ear	1966	88	8	9.1	6	6.8
	1967	63	6	9.4	6	9.4
Respiratory tract	1966	202	16	7.9	16	7.9
	1967	117	10	8.5	8	6.8
Urogenital tract	1966	110	6	5.5	6	5.5
	1967	96	9	9.4	6	6.3
Adult pyoderma, pyogenic infections of deeper tissues, septicaemia	1966	194	6	3.1	7	3.6
	1967	166	11	6.6	9	5.4
Superficial eye infections	1966	60	2	3.3	1	1.7
	1967	73	6	8.2	2	2.7
Infantile pyoderma	1966	133	1	0.8	—	0.0
	1967	66	—	0.0	—	0.0
Other infections	1966	74	1	1.4	1	1.4
	1967	36	7		4	
Total	1966	861	40	4.6	37	4.3
	1967	617	49	7.9	35	5.7

To elucidate the cause of this, it was observed that the incidence of resistant strains showed a periodical fluctuation, and that strains originated from the same ward or department were often identical in antibiotic sensitivity and biological properties. This finding indicated that the increasing incidence of resistant staphylococci should be attributed to their spreading from patient to patient in the same ward.

When staphylococci resistant to both methicillin and oxacillin began to appear, it was suggested that the cultures differed in some biological properties

Table II

Properties of Staph. aureus strains resistant and sensitive to methicillin and oxacillin

	No. of strains	Degree of activity	Biochemical activity				Pigment production	
			Haemolysis	Mannitol fermentation	Gelatin liquefaction	Urea hydrolysis	Yellow	White
Methicillin and oxacillin-resistant strains	89	+	80	70	72	12	83	17
		±	10	22	13	5		
		—	10	8	15	83		
Methicillin and oxacillin-sensitive strains	84	+	63	65	67	12	77	23
		±	21	25	24	7		
		—	16	10	9	81		

Note. Rounded off figures indicating percentage of strains.

+ = strong positivity; ± = weak positivity; — = negative.

from methicillin-sensitive strains [1, 16, 17]. In view of these findings we examined the biochemical reactions, phage susceptibility, population and growth properties of our strains. Table II shows a comparison of resistant and sensitive cultures as to behaviour in tests considered to indicate pathogenicity.

It is seen that resistant strains exerted a more frequent and stronger yellow pigment-producing, haemolysing and mannitol-fermenting activity than did the sensitive strains isolated from the same environment; the difference, however, was not significant statistically.

Table III shows the distribution of resistant strains according to phage groups and types. Strains belonging to phage group III and untypable cultures predominated among resistant staphylococci. Members of groups I, II and IV were also encountered. It should be noted that the cultures were highly resistant to the lytic effect of phages: 43% of the typable strains were lysed only on heating.

Table III*Phage group and type distribution of methicillin and oxacillin-resistant Staph. aureus strains*

Phage group	Number of strains	Types*
I	5	79 (3); 79/80 (2)
II	13	71
III	29	54 (23); 7/54 (3); 54/81 (2); 77AD (1)
IV	10	87
Mixed	4	3A/3C/55 (1); 53/83A/75 (1); 79/3C/75/54 (1); 79/3A/3C/55 (1)
Untypable	28	

* Bracketed figures mean the number of strains.

Population analysis was carried out with 10 strains on methicillin and on oxacillin blood agar plates. The antibiotics were incorporated in the agar media at concentrations allowing a good growth in broth of the examined strain.

Table IV*Population analysis of methicillin and oxacillin resistant Staph. aureus strains*

Strains	Number of colony-producing units per ml medium		
	Antibiotic-free broth	Methicillin broth	Oxacillin broth
5814/1966	1.6×10^9	4×10^4 (128 μ g)	9.4×10^4 (64 μ g)
634/1967	1.7×10^9	2.2×10^4 (128 μ g)	7.1×10^4 (64 μ g)
4082/1966	2.3×10^9	9.2×10^3 (128 μ g)	2.3×10^4 (64 μ g)
2306/1967	1.9×10^9	2.3×10^4 (64 μ g)	2.3×10^4 (64 μ g)
291/1967	1.3×10^9	4×10^4 (64 μ g)	3.3×10^4 (16 μ g)
4997/1967	5.7×10^8	1.3×10^4 (64 μ g)	9.1×10^3 (16 μ g)
2305/1967	1.1×10^9	10^5 (32 μ g)	2×10^4 (16 μ g)
1478/1967	1.9×10^9	3.2×10^4 (32 μ g)	3.6×10^4 (16 μ g)
100111 *	1.1×10^9	4.3×10^5 (128 μ g)	10^7 (64 μ g)
18 R**	1.1×10^9	6.7×10^8 (16 μ g)	5×10^8 (8 μ g)

* Strain received from DR. G. T. STEWART.

** Strain received from DR. MARY BARBER.

Table IV shows the number of colony-producing units per ml of a 24-hour culture.

Eight out of the examined strains were isolated in our laboratory. Strain 100111 (naturally resistant) and strain 18R (laboratory-induced) were kindly supplied by DR. G. T. STEWART and DR. MARY BARBER, respectively.

Four strains were subjected to population analysis on blood agar plates supplemented with 128 μg per ml of methicillin (3 strains of our collection and strain 100111). Our cultures contained on the average 1 resistant colony-forming unit among 79000 sensitive colony-forming units. For 3 strains examined in the presence of 64 μg per ml of methicillin, the frequency of resistant colony-forming units was 1/50000. For 2 strains a mean value of 1/23000 was obtained at 32 μg per ml of methicillin. The laboratory-induced resistant strain 18R consisted of an almost homogeneous population not susceptible to 16 μg per ml of methicillin. Five strains were analysed at an oxacillin concentration of 64 μg per ml. One of the cultures, strain 100111, differed considerably from our isolates which were characterized by a 1/35000 frequency of resistant colony-forming units. At 16 μg per ml concentration the mean incidence for 4 strains was 1/50000. Strain 18R, when tested on plates containing 8 μg per ml of oxacillin, comprised an almost homogeneous resistant population.

Thus, each naturally occurring resistant strain consisted of a mixed population. The overwhelming majority of the population was sensitive to both antibiotics. The distribution of resistant colony-producing units was not identical in the strains. The strain in which resistance had been induced artificially, in contrast to the naturally occurring resistant cultures, was characterized by a definitely homogeneous population.

On methicillin and oxacillin blood agar the resistant cells produced visible colonies only on the 2nd day. As compared to those on antibiotic-free medium the colonies were considerably smaller, more convex, less pigmented and different in size. On further incubation the haemolytic zone around antibiotic blood agar colonies was larger than the zone around simple blood agar colonies.

Growth curves for the strains were determined by the use of a biophotometer. Fig. 1 shows the growth curves for 2 naturally occurring resistant strains.

There was no difference between resistant and sensitive staphylococci in growth in antibiotic-free medium. In the presence of methicillin and oxacillin the shape of the growth curve was different. After a very long lag phase there was a logarithmic phase characterized by a less steep lag; the change from the logarithmic into the stationary phase was more gradual. As compared to the control culture, in the presence of the antibiotics the lag phase lasted 10 to 12 times, the logarithmic phase 2 to 3 times longer. In oxacillin medium the logarithmic phase curve was somewhat steeper than in methicillin medium. This type of growth curve, as shown in experiments with several strains, is characteristic of naturally occurring resistant strains consisting of mixed populations.

The growth curves for cultures in which resistance had been induced by serial passage in the presence of methicillin and oxacillin, considerably differ from the above type. Fig. 2 shows the growth curve for strain 18R.

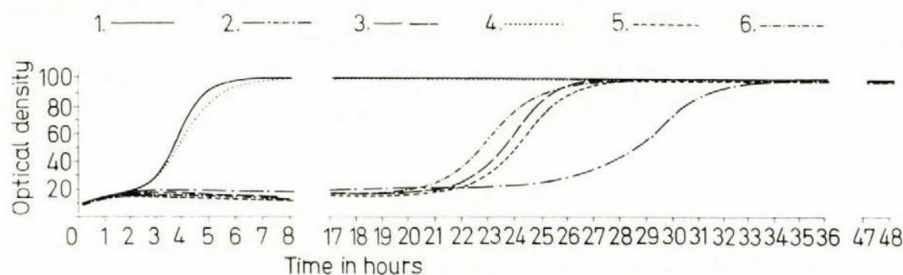


Fig. 1. Growth curves for *Staph. aureus* strains 4997/1967 and 5814/1966. Bonet Maury et Jouan biophotometer. 1 = strain 4997/1967, antibiotic-free medium; 2 = strain 4997/1967, methicillin, 32 $\mu\text{g/ml}$; 3 = strain 4997/1967, oxacillin, 16 $\mu\text{g/ml}$; 4 = strain 5814/1966, antibiotic-free medium; 5 = strain 5814/1966, methicillin, 128 $\mu\text{g/ml}$; 6 = strain 5814/1966, oxacillin, 64 $\mu\text{g/ml}$

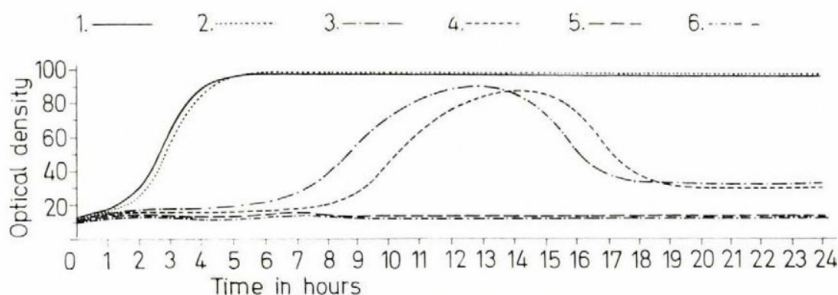


Fig. 2. Growth curves for *Staph. aureus* strain 18R. Bonet Maury et Jouan biophotometer. 1 and 2 = antibiotic-free medium; 3 and 4 = methicillin, 32 $\mu\text{g/ml}$; 5 and 6 = methicillin 64 $\mu\text{g/ml}$

All the curves obtained in the same medium were similar in shape. The lateral shift may primarily be attributed to slight differences in the viable cell count of the inoculum and in the antibiotic concentration of the medium. In repeated experiments the same type of multiplication was observed.

In the presence of methicillin the lag phase was considerably shorter for the laboratory-induced than for the naturally occurring resistant strain. The logarithmic phase lasted for about the same time, but in the stationary phase there was a marked difference between the two organisms. The optical density of cultures of the naturally occurring resistant strains remained unaltered in the stationary phase; in contrast, in the culture of the laboratory-induced resistant strain a rapid lysis was observed. On further incubation a secondary logarithmic phase began.

Discussion

Several observations indicate that the number of *Staph. aureus* strains resistant to methicillin and oxacillin is increasing. PARNAS and JABLONSKI [12] in 1961 were the first to report on the high incidence (14%) of methicillin-resistant staphylococci. In 1962 CHABBERT and BAUDENS [5] reported that 10 out of 82 strains were resistant to methicillin. In 1963 STEWART and HOLT [16] observed the spread of methicillin-resistant staphylococci in a paediatric department; during a 14-month period they isolated 71 resistant strains from 37 patients treated in 8 wards. In the next year COURTIEU *et al.* [7] described a 14%, PAL and GHOSH [11] a 11.8% incidence of methicillin-resistant strains. BOROWSKI *et al.* [4] isolated resistant bacteria in 50% from the nose of mothers and infants. In 1965 COLLEY *et al.* [6] showed that 79 out of 394 strains isolated in a general hospital were resistant to methicillin. ERIKSEN *et al.* [8] in 1966 demonstrated methicillin-resistance in about 10% of their strains. In Hungary the incidence of resistant strains ranged between 2.7 and 9% [2, 3, 10, 13–15, 18]. In our material the incidence of methicillin and oxacillin-resistant staphylococci was 4.3–4.6% in 1966 and 5.7–7.9% in 1967. Their occurrence was characterized by periodical peaks; the strains isolated from the same ward in the same period were frequently identical in methicillin and oxacillin-resistance, biochemical reactions and phage type. It is, therefore, probable that the resistant organisms were spreading from patient to patient.

Staph. aureus strains resistant to methicillin and oxacillin occurred in all phage groups. Members of phage group III and untypable strains predominated. The strains were highly resistant to the lytic effect of phages.

Naturally occurring methicillin and oxacillin resistant strains consist of mixed populations. In our strains the frequency of resistant colony-producing units was 1/230000 to 1/79000. SUTHERLAND and ROLINSON [17] found that the frequency of mutants resistant to 12 µg per ml of methicillin was 250 per 1 000 000.

In antibiotic-free liquid medium the resistant *Staph. aureus* strains multiply similarly to sensitive strains. In the presence of methicillin or oxacillin the growth curve is atypical, characterized by a protracted lag and logarithmic phase. According to the experiments of SUTHERLAND and ROLINSON [17] and GRAVENKEMPER *et al.* [9] the prolongation of the logarithmic phase is attributable partly to a longer generation time in resistant cells and partly to the fact that the dividing cells produce not only resistant but also sensitive bacteria which undergo a rapid lysis in the presence of the antibiotic. In methicillin medium there is a characteristic difference in the type of growth between naturally occurring and laboratory-induced resistant staphylococci.

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RELATIONSHIP BETWEEN RESISTANCE AND INACTIVATION OF METHICILLIN AND OXACILLIN IN STAPHYLOCOCCUS AUREUS

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Summary. Naturally occurring methicillin and oxacillin-resistant *Staph. aureus* strains characterized by heterogeneous population and penicillinase production were found to inactivate methicillin and oxacillin.

Inactivation of methicillin begins in the stationary phase of growth; the rate of destruction increases on further incubation. Oxacillin inactivation, in contrast, begins in the logarithmic phase.

The destruction of these antibiotics is not necessary for the growth of naturally occurring methicillin- and oxacillin-resistant staphylococci. Inactivation is a secondary process not associated with resistance.

Since the basic experiments of STEWART and HOLT [15] and ERIKSEN and ERICHSEN [6] who had shown that penicillinase-producing methicillin-resistant staphylococci inactivated isooxazole penicillins and all "penicillinase-stable semisynthetic" penicillins, many authors have focussed their attention on the mechanism of resistance to methicillin and oxacillin. Destruction of these antibiotics by resistant staphylococci has been confirmed [1, 7, 8, 10] and it has been suggested that the resistance may be due to this process.

Investigators not being able to demonstrate higher degrees of inactivation attributed the resistance to semisynthetic penicillins to an increased "intrinsic" resistance to these drugs [2, 5, 16, 18]. The possibility of this mechanism has not been denied by advocates of the former conception.

A third group of workers occupied a middle way in supposing that resistance to methicillin and oxacillin was a heterogenetic resistance deriving from an "intrinsic" factor and penicillinase production [3—5]. KAYSER [9] explained the "intrinsic" resistance by a genetic instability.

In a previous paper we have shown that methicillin- and oxacillin-resistant strains did not significantly differ in general biochemical properties from sensitive cultures. We have also demonstrated that the presence or absence of the antibiotic considerably influences the shape of the growth curve for resistant staphylococci [12].

In the present study answers were sought to the following questions: (1) Do staphylococcal strains resistant to methicillin and oxacillin and consisting of a mixed population inactivate these antibiotics in various phases of growth?

(2) Is methicillin and oxacillin destruction associated with the degree of resistance?

Materials and methods

Strains. Methicillin- and oxacillin-resistant strains were isolated from clinical materials [12].

Inactivation of methicillin and oxacillin. In 5 ml volumes of 0.5% glucose-Casitone (Difco) broth [17] two parallel rows of twofold serial dilutions ranging from 512 to 4 $\mu\text{g/ml}$ concentrations were prepared for both antibiotics. Inocula comprised 0.5 ml of a 24 hour broth culture diluted 1 : 100. Incubation lasted for 48 hours at 37°C. After 24 hours incubation the culture in one of the parallel rows showing abundant growth at the highest antibiotic concentration was passed through a G-5 filter. The filtrate was diluted serially so that the last tube contained an estimated antibiotic concentration of 0.06 $\mu\text{g/ml}$. After 48 hours the corresponding culture in the other row was similarly filtered and diluted serially. The serial dilutions were then inoculated with 0.1 ml aliquots of the 1 : 100 dilution of 24 hour broth cultures of penicillinase negative, methicillin- and oxacillin-sensitive *Staph. aureus* strains SG 511 and 100.

Controls were made by adding 0.5 ml medium to 5 ml volumes of methicillin and oxacillin solutions (32 $\mu\text{g/ml}$) and incubating the tubes at 37°C. The solutions were filtered after 24 and 48 hours and diluted serially to 0.06 $\mu\text{g/ml}$ concentration. The tubes were inoculated and read as described above.

The degree of inactivation was estimated from the decrease in antibiotic concentration in the test suspension as compared to the uninoculated control medium.

Two strains were examined for methicillin and oxacillin inactivation in various phases of growth. The strains were cultured in cuvettes of the Bonet Maury et Jouan biophotometer in the presence of antibiotics (methicillin 128 $\mu\text{g/ml}$ and oxacillin 64 $\mu\text{g/ml}$ for strain 5814/1966; methicillin 32 $\mu\text{g/ml}$ and oxacillin 16 $\mu\text{g/ml}$ for strain 4998/1967). At the end of the lag and the logarithmic phases and after 48 hours the content of one cuvette was filtered and the antibiotic concentration in the filtrate was assayed as described above. Control examinations were carried out in one cuvette filled with uninoculated antibiotic medium and in another containing antibiotic-free medium seeded with the appropriate cultures. Filtrates of the control cultures were also tested for bacteriostatic activity.

Penicillinase activity of cultures grown for 24 hours in the presence and absence of methicillin and oxacillin was determined by the iodometric method of PERRET [11]. Prior to inoculation the bacteria were passaged three times in the corresponding antibiotic-containing and antibiotic-free medium. The antibiotics were used at concentrations allowing abundant growth in 24 hours. Results were expressed as μM penicillin/ml culture/30 minutes.

The frequency of resistant colony-producing units was estimated as described previously [12].

Results

Table I summarizes the methicillin resistance, inactivating effect on methicillin, non-induced and induced penicillinase activity of methicillin-resistant staphylococci as well as the frequency of resistant cells in mixed populations.

None of the naturally occurring methicillin-resistant strains which had not been treated with methicillin inactivated the antibiotic in 24 hours, although all were able to multiply at the methicillin concentration used. After 48 hours all cultures were destroying methicillin. The laboratory-induced methicillin resistant culture, 18R, exerted no methicillin-inactivating effect.

Table II shows the results of oxacillin inactivation. The oxacillin-resistant strains which had not been induced with oxacillin and comprised a heteroge-

Table I

A comparison of methicillin-resistant Staph. aureus strains

Strains	Incubation, hours	Meth. M.I.C. $\mu\text{g/ml}$	Methicillin $\mu\text{g/ml}$		Penicillinase activity		Resistant cells, per thousand
			Initial	Inactivated	Non-induced	Induced	
1478/67	24	64	32	0	10.69	42.92	0.017
	48	128		16			
2305/67	24	64	32	0	14.16	37.96	0.091
	48	128		28			
2306/67	24	128	64	0	11.68	38.80	0.012
	48	256		40			
4997/67	24	128	64	0	12.87	44.51	0.023
	48	256		42			
291/67	24	128	64	0	9.70	44.26	0.031
	48	256		46			
4082/66	24	128	128	0	7.41	70.37	0.004
	48	256		64			
634/67	24	128	128	0	7.66	44.67	0.013
	48	256		96			
5814/66	24	128	128	0	11.63	52.01	0.025
	48	512		96			
100111*	24	256	128	96	11.38	106.10	0.391
	48	512		128			
18 R**	24	16	16	0	0.00	0.00	609
	48	64		0			

* Strain received from Dr. G. T. STEWART.

** Strain received from Dr. MARY BARBER.

The frequency of resistant cells was determined at the antibiotic concentration indicated in column 4.

neous population, destroyed about 50 to 100% of the antibiotic in 24 hours; by 48 hours they had eliminated the total amount of oxacillin from the medium. Laboratory-induced resistant strain 18R failed to inactivate oxacillin even in 48 hours.

Data of Tables I and II indicate that both methicillin and oxacillin are causing penicillinase production to increase. It may be assumed that these antibiotics are inactivated by penicillinase; this is supported by the fact that the resistant culture inactivates oxacillin more readily than it inactivates methicillin and it is known that oxacillin is much more sensitive to penicillinase

Table II

A comparison of oxacillin-resistant Staph. aureus strains

Strains	Incubation, hours	Oxac. M.I.C. $\mu\text{g/ml}$	Oxacillin $\mu\text{g/ml}$		Penicillinase activity		Resistant cells, per thousand
			Initial	Inactivated	Non-induced	Induced	
2305/67	24	48	16	8	11.68	49.22	0.012
	48	64		16			
4997/67	24	32	16	8	12.87	49.72	0.016
	48	128		16			
1478/67	24	32	16	12	10.38	41.43	0.019
	48	64		16			
291/67	24	32	16	12	9.70	41.78	0.025
	48	96		16			
4082/66	24	96	64	32	7.41	48.54	0.010
	48	192		63			
2306/67	24	64	64	40	14.16	41.43	0.018
	48	96		63			
634/67	24	64	64	48	7.66	51.85	0.042
	48	96		64			
5814/66	24	80	64	48	11.63	44.07	0.059
	48	320		64			
100111*	24	128	64	64	11.38	72.11	9.091
	48	256		64			
18 R**	24	4	8	0	0.00	0.00	454
	48	8		0			

* Strain received from Dr. G. T. STEWART.

** Strain received from Dr. MARY BARBER.

The frequency of resistant cells was determined at the antibiotic concentration indicated in column 4.

than methicillin [14]. The inactivated amount of antibiotic showed no direct proportion to the increase of the induced enzyme activity, as the penicillinase activity measured by PERRET's method [11] was not accompanied by a similar increase in antibiotic inactivation.

The data indicate that within the given range of antibiotic resistance (128 $\mu\text{g/ml}$, 64 $\mu\text{g/ml}$, etc.) those strains were inactivating higher amounts of the antibiotics which contained resistant cells in larger proportions.

In subsequent experiments, in order to determine the growth phase in which inactivation occurs, we examined the destruction of methicillin

Table III

Inactivation of methicillin and oxacillin by resistant Staph. aureus strains at the end of various growth phases

Strains	Initial concentration, $\mu\text{g/ml}$		Growth phase	Inactivated antibiotic, $\mu\text{g/ml}$	
	Methicillin	Oxacillin		Methicillin	Oxacillin
5814/66	128	64	lag	0	0
			logarithmic	0	32
			stationary	96	64
4997/67	32	16	lag	0	0
			logarithmic	0	12
			stationary	24	16

and oxacillin in cultures 5814/1966 and 4997/1967 at the end of the lag, logarithmic and stationary phases. Results are shown in Table III.

Lag phase cultures of resistant strains which had not been induced with methicillin or oxacillin, failed to inactivate the antibiotics. In the logarithmic phase of growth these cultures exerted no effect on methicillin, but inactivated oxacillin in 50 to 75%. Inactivation of methicillin began in the stationary phase and increased on further incubation.

The above results indicate that the inactivation of methicillin and oxacillin was not directly associated with resistance. In order to show that the degree of resistance determined *in vitro* depends on the proportion of resistant cells, the following experiments were performed. A mixed population and a resistant population which had been selected from strain 5814/1966 were cultured in cuvettes of the biophotometer. At various stages of the logarithmic phase uniform aliquots of methicillin were added to the cultures. Fig. 1 demonstrates the growth curve for the mixed population.

It is evident from Fig. 1 that methicillin exerted the same effect at the early, middle and late stages of the logarithmic phase. Due to a partial lysis of the bacteria the optical density of the culture decreased 30 to 60 minutes after the addition of the antibiotic. Then, indicating a long lag phase, the optical density remained identical for a long period of time. Multiplication started again after 16 to 19 hours. The cause of this was that the sensitive cells were lysed and the resistant cells needed a long adaptation time before they had become able to multiply. No destruction of the antibiotic was detected after 24 hours.

Fig. 2 shows the influence of methicillin on the growth curve for the selected resistant population. When the antibiotic had been added at the beginning of the logarithmic phase, multiplication ceased and optical density decreased.

ed; after a 6 hour interval growth started again and was characterized by a protracted logarithmic phase. In the next stages of growth the curve showed the usual shape. When methicillin had been added in the middle of the logarithmic phase, 3 hours after the beginning of multiplication, growth discontinued for

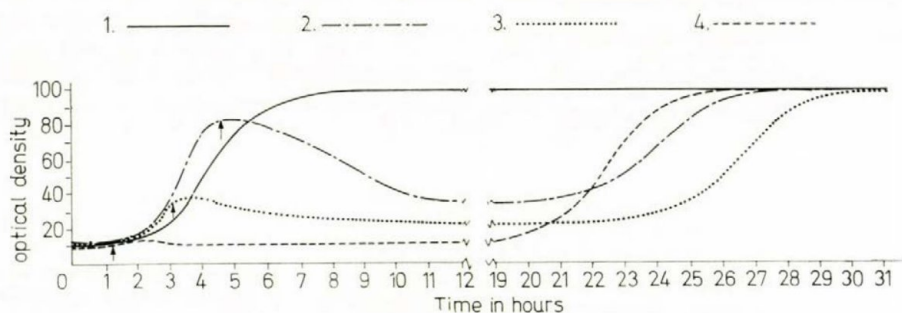


Fig. 1. Changes in the growth curve for *Staph. aureus* strain 5814/1966 under the effect of methicillin added in various stages of the logarithmic phase. 1 = growth in methicillin-free medium; 2 = growth in the presence of 64 $\mu\text{g}/\text{ml}$ of methicillin added 3 hours after the beginning of the logarithmic phase; 3 = growth in the presence of 64 $\mu\text{g}/\text{ml}$ of methicillin added 1½ hours after the beginning of the logarithmic phase; 4 = growth in the presence of 64 $\mu\text{g}/\text{ml}$ of methicillin added at the beginning of the logarithmic phase

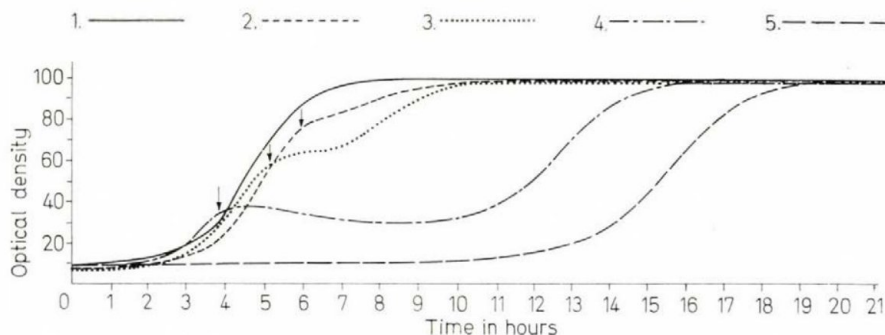


Fig. 2. Changes in the growth curve for a resistant population of *Staph. aureus* strain 5814/1966 selected on methicillin medium (128 $\mu\text{g}/\text{ml}$) under the effect of methicillin added in various stages of the logarithmic phase. 1 = growth in methicillin-free medium; 2 = growth in the presence of 64 $\mu\text{g}/\text{ml}$ of methicillin added 4 hours after the beginning of the logarithmic phase; 3 = growth in the presence of 64 $\mu\text{g}/\text{ml}$ of methicillin added 3 hours after the beginning of the logarithmic phase; 4 = growth in the presence of 64 $\mu\text{g}/\text{ml}$ of methicillin added 2 hours after the beginning of the logarithmic phase; 5 = growth in the presence of 64 $\mu\text{g}/\text{ml}$ of methicillin

1½ hours but no lysis occurred. Addition of methicillin 4 hours after the beginning of multiplication caused but a slight modification in the growth curve. No inactivation of the antibiotic was detected at the beginning of the second multiplication phase. When the antibiotic medium had been inoculated with a 24 hour culture of the selected strain diluted 1 : 100, the lag phase lasted for 12 hours and subsequent multiplication was characterized by a protracted logarithmic phase observed usually with methicillin-resistant cultures.

Discussion

At suitable concentrations, both methicillin and oxacillin are inactivated by naturally occurring resistant *Staph. aureus* strains. While oxacillin is inactivated by multiplying and stationary phase cultures, methicillin inactivation takes place only in the stationary phase.

STEWART and HOLT [15], ERIKSEN and ERICHSEN [7] and GRAVENKEMPER *et al.* [8] have shown a well-defined methicillin inactivation in 24 hours and oxacillin inactivation in 6 hours, because they had eliminated the sensitive cells, shortened the adaptation time and increased the inactivating capacity of the cultures by induction with methicillin. Moreover, they applied low antibiotic concentrations and large inocula.

Under the effect of methicillin and oxacillin induction, the cultures' penicillinase activity increases several times. It is evident that the degree of the hydrolysis of methicillin and oxacillin depends on the sensitivity of these antibiotics to staphylococcal penicillinase. SHNERSON [14] showed that to penicillinase activity in living cultures methicillin was 100 000 times and oxacillin was 500 times more resistant than benzylpenicillin. YURCHENCO *et al.* [18] demonstrated in experiments in vitro and in vivo that even at high concentrations purified staphylococcal penicillinase failed to destroy methicillin.

Despite the numerous investigations into this problem the mechanism of methicillin resistance in *Staph. aureus* is unknown. The examinations have mainly been focussed on the inactivation of the antibiotic and its mechanism. Our results agree with data described in the literature, we have confirmed that penicillinase-producing naturally occurring resistant staphylococci inactivate methicillin and oxacillin. Destruction of the antibiotics is due to the penicillinase activity of the culture [7, 8, 13, 15]. Although penicillinase negative methicillin-resistant strains are capable of growing in the presence of methicillin, they are unable to inactivate the antibiotic. These observations are not sufficient for explaining the mechanism of the development of resistance. Our experimental data indicate that the biological changes which enable the resistant cells to multiply in the presence of the antibiotic occur during a very long lag phase. The nature of these changes is unknown. The fact that the young multiplying culture exhibits an increased sensitivity to the antibiotic indicates that the alterations ensuring its ability to multiply occur in a stage preceding the phase of active multiplication. Inactivation of the antibiotic is a secondary phenomenon which is not the cause but the consequence of resistance.

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REGULATION OF ALKALINE PHOSPHATASE PRODUCTION IN THE AUXOTROPHS OF *BACILLUS ANTHRACIS*

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Summary. In prototrophic and auxotrophic strains of *Bacillus anthracis* three different types of repressible alkaline phosphatase production have been distinguished. Type 1 is characterized by a repression increasing gradually with the concentration of orthophosphate. This type of repression was observed in prototrophs, in some pyrimidine and vitamin-dependent auxotrophs, and in purine auxotrophs blocked before the closing of the purine ring. Type 2 is represented by adenine-dependent auxotrophs deficient in adenylosuccinase or adenylosuccinase lyase. Type 2 repression occurs in two phases. Four out of 5 guanine-dependent strains displayed repression type 3 characterized by a complete inhibition of alkaline phosphatase production even at minimum phosphate concentrations allowing suboptimal growth. Reversion to prototrophy restored the original repression type characteristic of the parent strain. The regulation of alkaline phosphatase formation seems to be associated with the biochemical pathway of purine synthesis, that is, the production of this catabolic enzyme is connected with the metabolic phases of purine synthesis.

Alkaline phosphatase production in *Bacillus anthracis* and *B. cereus* is repressed by inorganic orthophosphate [1]. The degree of repression is especially high in *B. anthracis*. We isolated a considerable number of *B. anthracis* auxotrophs [2] and have shown that mutation in the purine loci may cause an increase or a decrease in the repression of alkaline phosphatase production. Accordingly, a mutation in a structural gene may alter the regulation of the production of an enzyme independent of the affected locus. The present paper gives an account of this observation.

Materials and methods

Bacterial strains. Isolation and characterization of *B. anthracis* auxotrophs were described previously [2, 3]. The mutants were obtained from the non-capsulogenic variant of *B. anthracis* strain Vollum (VC⁻). Instead of the previous designation [2, 3], the auxotrophs were termed on the basis of the recommendation of DEMEREC *et al.* [4]. The nomenclature of these authors could not be applied to all cultures, determination of the genetic structure being impossible without recombination experiments. As no such methods are known for *B. anthracis*, designations not described by DEMEREC *et al.* (*ade*, *gua*, *ura*) were also used for the grouping of phenotypes.

The organisms and their properties are listed in Table I.

The cultures were stored at 4°C as spore suspensions prepared with distilled water.

Culture media. The medium used in our previous work was modified to contain less phosphate. After dissolving 5 g lactalbumin hydrolysate (Difco) in 100 ml distilled water the solution was shaken for two hours with 10 g granulated Dowex-1 HCl. After subsequent filtration the volume was made up to approximately 1 litre. In the filtrate about 1/4 of the original

Table I
List of strains

Serial number of isolates	Designation of the strain	Remarks	Serial number of isolates	Designation of the strain	Remarks
	VC ⁻ , prototrophic	Parent strain of mutants	31	<i>pur-2</i>	
	Davis, prototrophic	Non-sporulating	62	<i>pur-3</i>	
			63	<i>pur-4</i>	
			58	<i>pur-5</i>	
6	<i>ade II-1</i>	See 1.	58a	revertant of <i>pur-5</i>	Prototrophic
20	<i>ade II-2</i>				
23	<i>ade II-3</i>		12	<i>ura-1</i>	See 5.
23a	revertant of <i>ade II-3</i>	Prototrophic	22	<i>ura-2</i>	
214	<i>ade II-4</i>		191	<i>ura-3</i>	
341	<i>ade I-1</i>	See 2.	60	<i>ura-4</i>	
344	<i>ade I-2</i>		177	<i>thy-1</i>	See 6.
30	<i>gua-1</i>	See 3.	102	<i>thy-2</i>	
2	<i>gua-2</i>		28	<i>rib-1</i>	See 7.
3	<i>gua-3</i>		26	<i>pab-1</i>	See 8.
13	<i>gua-4</i>		37	<i>bio-1</i>	See 9.
52	<i>gua-5</i>		4	<i>bio-2</i>	
61	<i>pur-1</i>	See 4.			

Note to Table I. The genotype (locus) of adenine mutants was determined in enzymologic experiments [5]. The phenotypic and genotypic characters of the strains were as follows: (1) *ade II* strains required adenine specifically and were deficient in adenylosuccino lyase (EC 4.3. 2.2); (2) *ade I* strains were deficient in adenylosuccino synthase (EC 6.3.4.4); (3) *gua* strains required guanine specifically; due to a block in the conversion of xanthine to guanylic acid they did not utilize xanthine; (4) *pur* strains utilized hypoxanthine, xanthine, guanine or adenine; they were deficient in an enzyme acting before the closing of the purine ring; (5) *ura* strains utilized uracil; (6) *thy* strains required thymine only above 32°C; at lower temperatures they behaved as prototrophs; (7) *rib* strains required riboflavin; (8) *pab* strains required paraaminobenzoic acid; (9) *bio* strains required biotin. Mostly non-capsulogenic forms were examined; in some experiments both non-capsulogenic and capsulogenic variants were used. The capsulogenic variants were isolated from non-capsulated forms as described in reference 3.

phosphate concentration was present. The lactalbumin hydrolysate medium was supplemented with L-glutamic acid, 2 g; KCl, 5 g; Na₂SO₄ (dehydrated), 1 g; DL-tryptophan, 0.1 g; L-cystine, 0.1 g; tris (hydroxymethyl) aminomethane, 4 g. The pH was adjusted with HCl to 7.2 and the volume was made up to 2000 ml. The medium was distributed into flasks and autoclaved at 110°C for 30 minutes. After cooling the following ingredients were added: ferric ammonium citrate, 5 µg; MnSO₄ · 4H₂O, 2.5 µg; MgSO₄ · 7H₂O, 100 µg; thiamine, 1 µg; glucose, 2 mg per ml.

For the cultivation of auxotrophs the medium was supplemented with purine or pyrimidine bases (10 µg/ml) or vitamins (1 µg/ml).

Cultivation. In the presence of 0.17 mM orthophosphate, which remained in the medium as an impurity of the lactalbumin hydrolysate, both wild-type and auxotrophic strains showed a boptimal growth.

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A series of media containing graded phosphate concentrations (0.37, 0.57, 0.87, 1.17, 1.37 and 1.67 mM) was prepared by adding appropriate amounts of a 20 mM K_2HPO_4 solution. In these media the bacteria were cultured and the bacterial suspensions used for alkaline phosphatase determination. It should be noted that no optimal growth was obtained at 0.37 mM phosphate concentration. The medium was distributed in 10 ml amounts into 100 ml Erlenmeyer flasks, seeded with spores and shaken at 37°C for 16 hours on a Gyrotory shaker operating at 240 r. p. m.

Quantitative determination of alkaline phosphatase. A 10 ml amount of the culture was centrifuged, washed with saline then resuspended in saline so as to give an optical density of 0.6 in a Bausch and Lomb Spectromic 20 photometer at 620 m μ wavelength. The suspension contained 0.32 mg per ml of dry bacteria.

To 2 ml bacterial suspension 2 ml reagent (0.5% p-nitrophenyl phosphate dissolved in pH 8.5 0.2 M Tris buffer) were added and the mixture was incubated in a water bath at 37°C for 20 minutes. Optical density was then determined in a Spectromom Model 201 photometer at 410 m μ wavelength. Enzyme activity was expressed as the amount of paranitrophenol released in 20 minutes.

In some experiments parallel determinations were performed with suspensions treated with toluol. The values for the treated suspensions were 15 to 20% higher than those for untreated bacteria. However, toluol exerted no effect on the type of repression. Unless otherwise indicated, the results refer to experiments with untreated suspensions.

Results

When the organisms were grown at various phosphate concentrations certain auxotrophs differed from the prototrophic strain in alkaline phosphatase activity. It should be emphasized that all prototrophs had derived from strain VC⁻. According to the repression of phosphatase production the strains were divided into three types.

Fig. 1 indicates that the repression of phosphatase production in prototrophic strain VC⁻ was at orthophosphate concentrations slightly lower than 0.57 mM. At higher concentrations the repression was considerable: in the range of 0.57–0.87 mM it amounted to more than 50%. A further increase in phosphate concentration did not markedly decrease the enzyme activity; the lowest values were obtained at a concentration of 1.17 mM. This kind of reaction was named *repression type 1*.

An example of *repression type 2* is demonstrated on auxotroph *ade II*–3. This type was characterized by a slight repression in the range of 0.57–0.87 mM phosphate; the repression remained at the same level up to 1.37 mM. At higher phosphate concentrations the activity decreased to a minimum.

Accordingly, as compared to the parent strain, culture *ade II*–3, in which the mutation had affected a well-defined structural gene responsible for adenylosuccino lyase production, showed an alkaline phosphatase repression different from type 1. That the change was due to a mutation in the above locus was indicated by the fact that the reversion of *ade II*–3 to prototrophy resulted in the restoration of a high degree of repressibility (Fig. 1).

Repression type 3 is represented in Fig. 1 by auxotroph *gua*-4.

Most auxotrophs yielded the type 1 curve characteristic of prototrophs. These strains are presented in Table II, where in addition to the maximum

activity level observed at 0.37 mM phosphate content the minimum values obtained at 1.67 as well as the concentration range in which repression was higher than 50% are shown. The latter value, with some exception (*ura-2*, *gua-5* and *pab-1*), was between 0.57 and 0.87 mM, though repression was already considerable in the range of 0.37–0.57 mM. This was probably due to the fact that even the smallest phosphate concentration exerted a partial repressive effect.

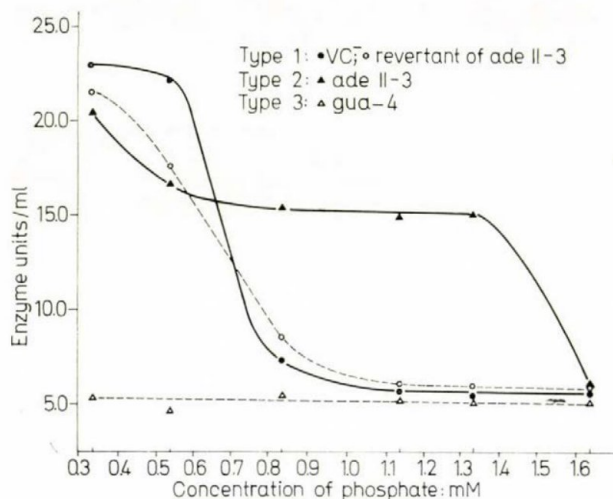


Fig. 1. Different types of the repression of alkaline phosphatase production

Strains characterized by a diphasic repression belonged to type 2. After an initial slight repression the formation of phosphatase remained at the same level in a wide range of phosphate concentration (0.57–1.37 mM) then decreased rapidly to a minimum value.

Inosinic acid is converted into adenylic acid by two enzymes: adenylosuccino synthase and adenylosuccino lyase. Upon losing either of these enzymes the type of alkaline phosphatase repression changes from type 1 to type 2. Both capsulogenic and non-capsulogenic variants of culture *ade II-3* behaved in this manner; prototrophic reversion of the organism was accompanied by a restoration of the original repression type. Repression type 2 curves were obtained also for other adenine-dependent strains (*ade I-1*, *ade I-2*, *ade II-2* and *ade II-4*).

The characteristic behaviour of adenine-dependent strains could not be attributed to the presence of adenine (10 µg/ml) in the medium. No change in repression type was observed when strain *VC⁻* had been cultured in a medium supplemented with adenine.

Type 3 was characterized by a very low degree of enzyme production which cannot be influenced appreciably by changing the concentration of

Table II
Strains displaying type 1 alkaline phosphatase repression

Strain	Remarks	Alkaline phosphatase activity (units)		Range of phosphate concentration (mM) with repression above 50%
		maximum values	minimum values	
VC ⁻		22.9	4.7	0.57—0.87
VC	Streptomycin-resistant	34.4	3.8	0.57—0.87
Revertant from <i>ade</i> II-3	prototrophic and capsulogenic	20.3	4.0	0.57—0.87
Revertant from <i>ade</i> II-3	prototrophic	21.4	5.3	0.57—0.87
<i>pur</i> -1		19.1	7.9	0.57—0.87
<i>pur</i> -2		27.3	7.3	0.57—0.87
<i>pur</i> -3		25.0	6.1	0.57—0.87
<i>pur</i> -4		23.8	6.1	0.57—0.87
Revertant from <i>pur</i> -5	prototrophic	21.3	6.0	0.37—0.57
<i>gua</i> -5		15.3	3.8	0.37—0.57
<i>ura</i> -1		24.4	3.8	0.57—0.87
<i>ura</i> -2		15.3	4.1	0.37—0.57
<i>ura</i> -3		30.8	6.1	0.57—0.87
<i>ura</i> -4		28.2	6.1	0.57—0.87
<i>thy</i> -1	37°C*	21.4	3.5	0.57—0.87
<i>thy</i> -1	26°C**	23.2	4.7	0.57—0.87
<i>thy</i> -2	37°C	24.4	5.0	0.57—0.87
<i>thy</i> -2	26°C	21.1	5.8	0.57—0.87
<i>bio</i> -1		17.5	5.3	0.57—0.87
<i>bio</i> -2		17.3	3.5	0.57—0.87
<i>rib</i> -1		20.0	6.4	0.57—0.87
<i>pab</i> -1		16.7	4.1	0.37—0.57

* grown at 37°C in the presence of thymine (10 µg/ml).

** grown at 26°C in the absence of thymine.

phosphate. The activity in toluol-treated suspensions was not significantly higher than in untreated suspensions; accordingly, this type of repression was not due to a change in permeability. The degree of activity corresponded to the minimum values obtained for types 1 and 2. The present experiments supplied no answer as to whether the very low value was to be attributed to a residue of a considerably repressed alkaline phosphatase activity or due to other phosphoric monoester hydrolases.

Type 3 included most guanine-dependent strains (*gua*-1, *gua*-2, *gua*-3 and *gua*-4). Isolate *gua*-5 was an exception, as it belonged to type 1 (Table II). This

striking behaviour of *gua-5*, an auxotroph identical in phenotype with the rest of the guanine-dependent cultures, cannot be explained.

Strain *pur-5*, in which the early stage of purine synthesis was blocked, also belonged to type 3; its revertant behaved as type 1 cultures.

Discussion

The alkaline phosphatase of *E. coli*, which is produced on the bacterial surface between the cell wall and the cytoplasmic membrane [6, 7], acts in the environment of the organism as a catabolic enzyme. For economic reasons the production of this enzyme is highly influenced by the presence of orthophosphate. At sufficient phosphate concentrations there is no need for an enzymatic breakdown of phosphate esters. Accordingly, in media with high phosphate concentrations alkaline phosphate production by wild-type *E. coli* strains is slight or nil [8, 9]. Repression of this exoenzyme has been reported in other microbial species such as *B. subtilis* [10], *Staphylococcus aureus* [11] and *Neurospora crassa* [12].

Alkaline phosphate production in *B. anthracis* and *B. cereus* depends similarly on the environmental phosphate concentration [1]. This finding should be taken into consideration when phosphatase production is used as a character for the classification of the two phylogenetically closely related organisms [13]. *B. cereus*, which exerts a strong phosphatase activity, is less sensitive to repression than the weak phosphatase-producing *B. anthracis* [1].

Alkaline phosphatase production in *E. coli* is associated with three genes. One of them (P) is responsible for the synthesis of the enzyme; two genes (R_1 and R_2) play a regulatory role. In wild-type *E. coli* strains the information of R_1 and R_2 genes represses the enzyme production proportionally with the phosphate concentration. Mutation in the two regulatory genes causes a condition in which the presence of phosphate fails to influence the enzyme production, that is, the mutation gives rise to constitutive mutants.

In part of the auxotrophs isolated from prototrophic *B. anthracis* strain Vollum the regulation of alkaline phosphatase production was different from that in the parent strain. The concentration of orthophosphate influenced the formation of the enzyme in three different manners. In prototrophic strains (type 1) the regulation of alkaline phosphatase production was independent of the loci responsible for capsule production and streptomycin sensitivity. Blocking of biochemical pathways regulating the synthesis of pyrimidine bases (uracil, thymine) or mutation to vitamin dependence exerted no effect on the type of repression. In contrast, mutation in genes responsible for purine synthesis may alter the regulation of alkaline phosphatase production. No change

was observed in 4 out of the 5 purine auxotrophs in which mutation had resulted in a block before the closing of the purine ring. There are no data for the genotype of our *pur* strains, but cross-feeding experiments indicate that two different kinds of deficiency exist (unpublished observation).

In adenine-dependent *B. anthracis* auxotrophs the regulation of alkaline phosphatase production was always different from that in the parent strain. Mutation in either of the two genes regulating the conversion of inosinic acid to adenylic acid caused a change in the type of alkaline phosphatase repression.

In auxotrophs characterized by a block in the conversion of xanthylic acid to guanylic acid there was also a change in the regulation of alkaline phosphatase production: except for one strain, a maximum repression was observed at very low phosphate concentrations.

The other two types of repression were characterized by a similar fall of enzyme production to low levels if phosphate was present at high concentrations. These findings indicate that more than one — at least two — regulating systems are involved in alkaline phosphatase production by *B. anthracis*. These systems are closely related with the structural genes for purine synthesis. A mutation in these genes causes an alteration in the regulation of alkaline phosphatase production. The same pleiotropic effect is observed when an auxotroph reverts to prototrophy: the revertant exerts the original type of enzyme regulation.

Our observations present an interesting example of the dual effect of the change in a structural gene. Similar observations have been reported for *E. coli* in which a structural gene mutation in the L-arabinose and galactose operon was influencing the function of other genes. If a mutation occurs in the locus for one of the three enzymes responsible for L-arabinose fermentation (kinase, isomerase and epimerase), the two other enzymes will be produced in increased amounts [16, 17]. Similar findings were revealed for the galactose operon. As an effect of a mutation in the galactose-kinase locus, the originally inducible other two enzymes (epimerase and transferase) become constitutive in character [18].

There is an essential difference between these findings on *E. coli* and our observations. The mutation of one structural gene in *B. anthracis* effects the regulation of an other, functionally different structural gene. Mutation in the genes regulating purine biosynthesis results in a change in the production of alkaline phosphatase, a catabolic enzyme. Recently an interesting observation has been reported concerning the association between extracellular protease production and sporulation [19]. In *B. cereus* these properties seem to be regulated by two repressor systems which are connected directly or indirectly with numerous biochemical pathways. Thus the degree of protease production is associated with mutations in the purine and pyrimidine loci. The reversion of such auxotrophs to prototrophy restores the original properties. In the two

related species, *B. cereus* and *B. anthracis*, the regulation of the formation of catabolic exoenzymes is probably influenced by several other metabolic pathways.

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THE ULTRASTRUCTURE OF VACCINIA VIRUS GROWING IN THE EMBRYONATED EGG

By

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(Received, January 8, 1968)

Summary. The multiplication of vaccinia virus in chorionic cells of the chick embryo chorio-allantoic membrane was studied by electron microscopy.

It was found that virions adsorb to chorionic cells without orientation and are engulfed like foreign bodies. Destruction of the virion's outer membrane system and the release of the core take place in the period when free infectious virus is at the lowest level. Subsequently the characteristic virus-producing matrix (virus factories) and immature virus particles are well demonstrable in juxtannuclear position. The particles are gradually shifted towards the periphery of the cytoplasm, where transient and mature forms soon appear.

Thus, all the developmental forms described for infected cultured cells are demonstrable in the infected chorionic cells.

Consequently, for morphological investigations the embryonated egg is as suitable as are the tissue culture systems.

The morphological events taking place in the course of the reproduction cycle of vaccinia virus were studied in the earliest time of research in ultrathin sections prepared from the chorio-allantoic membrane (CAM) of the chick embryo [1–4]. Being simple systems, cell cultures have recently been favoured [5–11], often combined with microautoradiography [12–14]. Thus the different stages of the vaccinia virus reproduction cycle have been characterized in cell culture systems.

In the present work we have returned to the CAM system. First, we have taken into consideration that the recently developed methods might enable us to follow viral reproduction in the chorionic cells as well. Secondly, we are planning to study the antiviral effect of methylisatine- β -thiosemicarbazone, and for this purpose we found the CAM superior to cell cultures [18]. It is a further advantage of the embryonated egg that the antiviral substance can be injected into the yolk sac, which is not in direct contact with the chorionic cells. Thus, for measuring antiviral activity the embryonated egg presents a system similar to the living animal.

Materials and methods

Virus. The CAM-adapted variant of the Budapest vaccine strain was used throughout. Purified virus suspension containing $10^{6.2}$ PFU per ml was suspended, and stored in skimmed milk at -20°C [15]. Artificial air-sac was prepared in 11-day embryonated eggs according to NADEJE's technique [16]. In three experiments 100,000, 1000 and 100 PFU, respectively,

were dripped on to the CAM, in 0.2 ml throughout. Especial care was taken to distribute the inoculum on the CAM homogeneously. The eggs were re-incubated at 37°C. Four eggs each, were opened 3, 6, 12, 24 and 48 hours after inoculation. These were cut into two pieces in the horizontal plane of the longitudinal axis. The allantoic sac was left attached to the upper half of the egg shell. Peripheral parts of this half were cut off until the detached part of the CAM, the part limiting the artificial air-sac, had separated. The CAMs were rinsed three times with sterile saline and carefully halved.

Electron-microscopic preparation. One half of each CAM was immediately pre-fixed at 4°C in 4% glutaraldehyde dissolved in 0.15 M phosphate buffer of pH 7.4, allowed to stand for 2 to 24 hours, washed and post-fixed in an OsO_4 solution buffered as recommended by CAULFIELD [20]. For embedding methyl- and butylacrylate or Araldite was used. Ultrathin sections were cut with a Porter-Blum or LKB microtome and prepared on micro-screens devoid of membrane. The sections embedded in metacrylate and Araldite were counter-stained with lead citrate for 10 minutes and 20 minutes, respectively. Micrographs were taken in a Hitachi-HU-10A electron microscope, using 75 KV accelerating voltage.

Virus titration. The unfixed half of each membrane was weighed in the wet state, ground in a mortar with quartz sand and diluted in saline to make a 10% suspension. Serial tenfold dilutions of this were inoculated into four embryonated eggs each, within several hours. The eggs were opened 48 hours later and the plaques on the CAMs were counted.

Results

The reproduction curves calculated from the infectivity values of the CAMs infected with three different doses of vaccinia virus are presented in Fig. 1.

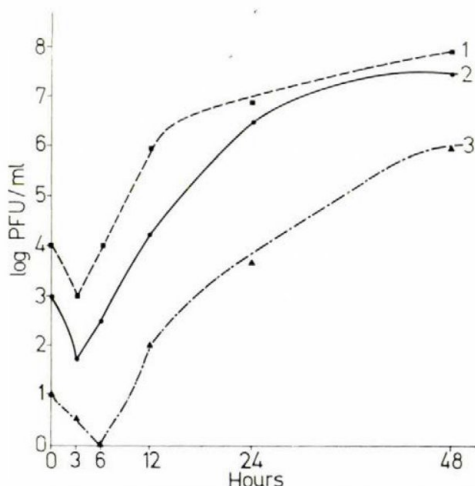


Fig. 1. Multiplication of vaccinia virus in the CAM of the embryonated egg. Eleven-day old embryonated eggs were inoculated through an artificial air-sac with 100 (3), 1000 (2) and 100,000 PFU of vaccinia-virus. At each of the indicated times from four eggs the part of the CAM bordering the artificial air-sac was cut off and halved. From one half a 10% suspension was prepared and titrated, using a tenfold dilution series, and four embryonated eggs per dilution.

The titre calculated from the 48-hour plaque count is plotted in the Figure

The preparations obtained from the CAMs infected with the smallest dose failed to illustrate the morphological events satisfactorily.

The electronmicrographs shown represent CAMs infected with 1000 or 100,000 PFU. The difference between these doses did not manifest itself in a significant morphological difference. Even the reproduction curves ran parallel.

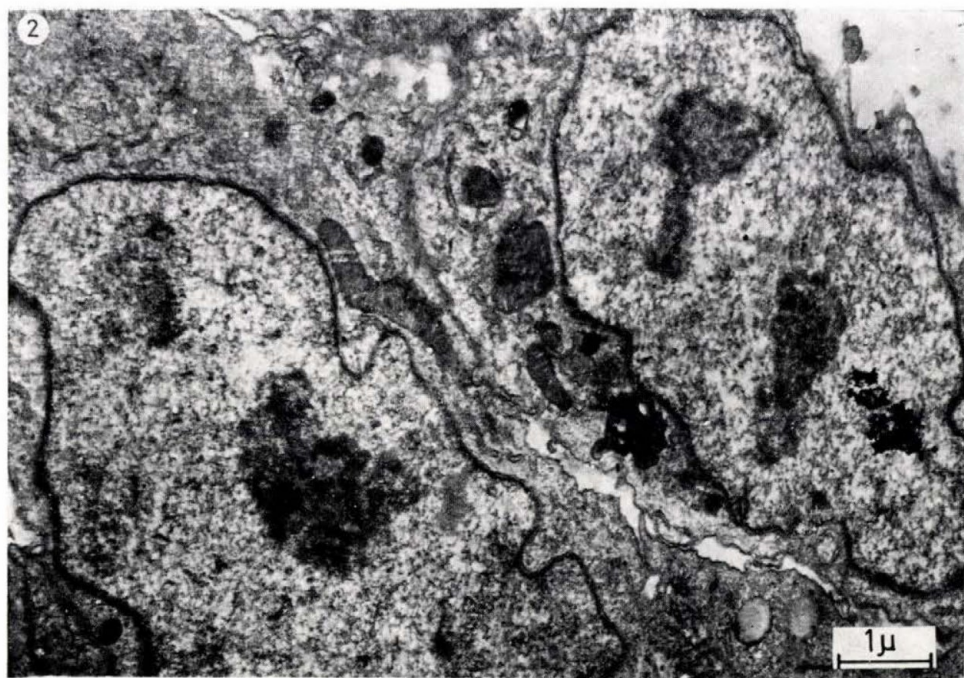
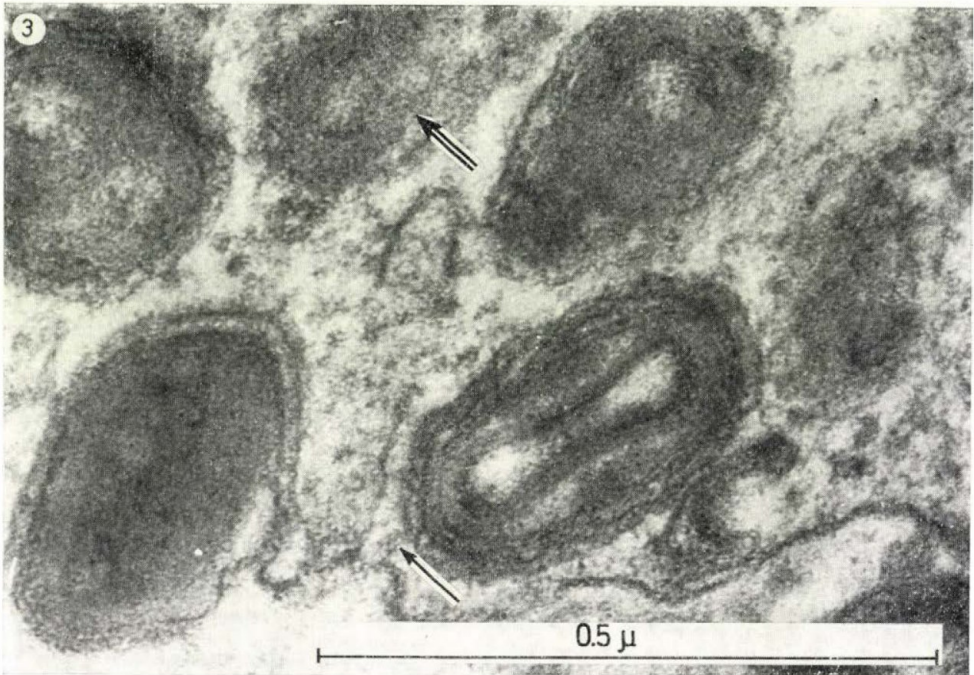


Fig. 2. Chorionic cells from uninfected 13-day embryonated eggs (Photo No. 7211) $\times 11,400$

The logarithmic phase of reproduction started between 3 and 6 hours postinoculation. After 24 hours the slope of the curve began to decline, but the rise of infectivity was still significant during the second 24-hour period. The photos representing the initial phases (adsorption, penetration, uncoating) show chorionic cells from embryos infected with 100,000 PFU 3 hours before fixation. The cells representing the logarithmic and late phases are from eggs infected with 1000 PFU and fixed 6, 12, 24, or 48 hours postinoculation.

Fig. 2 is an electronmicrograph of chorionic cells of the uninfected 13-day CAM. The mostly polygonal cells are approximately identical in structure with the corresponding cells of the 11-day embryo. They are close to one another. The nucleus, having one or two nucleoli, fills a relatively large part of the cell. The cytoplasm shows no peculiarity, all the organelles characteristic of the animal cell are to be found in it.

Viral adsorption, penetration and uncoating. The chorionic cells shown in Figs 3–5 are from CAMs infected with 100,000 PFU 3 hours before fixation. The virions on the cell surface show no orientation. In Fig. 3 a virion cut in its widest section plane is partly enclosed by the cell membrane. At the



Figs 3–5. Adsorption, penetration, uncoating

Fig. 3. The virion in the right has penetrated the cell. It is surrounded by a reduplication of the cell membrane, which is at the arrow a direct continuation of the outer wall. The double arrow points to a particle turning into the eclipse. Digestion of the cell membrane and of the virion's own membrane as well as the early stage of the release of the core are well noticeable (Photo No. 9216) $\times 165,000$

site of their attachment the membranes of both the virion and cell are accentuated. In the nucleoid with indistinct limiting membrane a filamentous structure is visible. The nucleoid is surrounded by a thick layer of viroplasm, which is the thinnest, 130–150 Å, at the two poles of the virion. Its greatest thickness is 270 Å. The other virions, cut in different planes, have already penetrated the cell. Three of them are still surrounded by the membrane systems. The one cut in the profile plane parallel to its longitudinal axis is still connected with the outer membrane of the cell (arrow). In this virion the structure characteristic of the mature virion is clearly visible; the disc-like nucleoid is impressed at its centre. Its double membrane, the structured limiting membrane of the virion as well as the lateral bodies are well discernible. The virion in the left upper corner is cross-sectioned, a third one is cut

in an oblique plane. In the fourth virion engulfed in the cell (double arrow) enzymatic digestion of the virion's outer membrane as well as uncoating of the DNA-containing core has started.

Mature virions were found to measure 250–300 $m\mu$ in length, 200–250 $m\mu$ in width and 150–200 $m\mu$ in height. The outer membrane is 100–

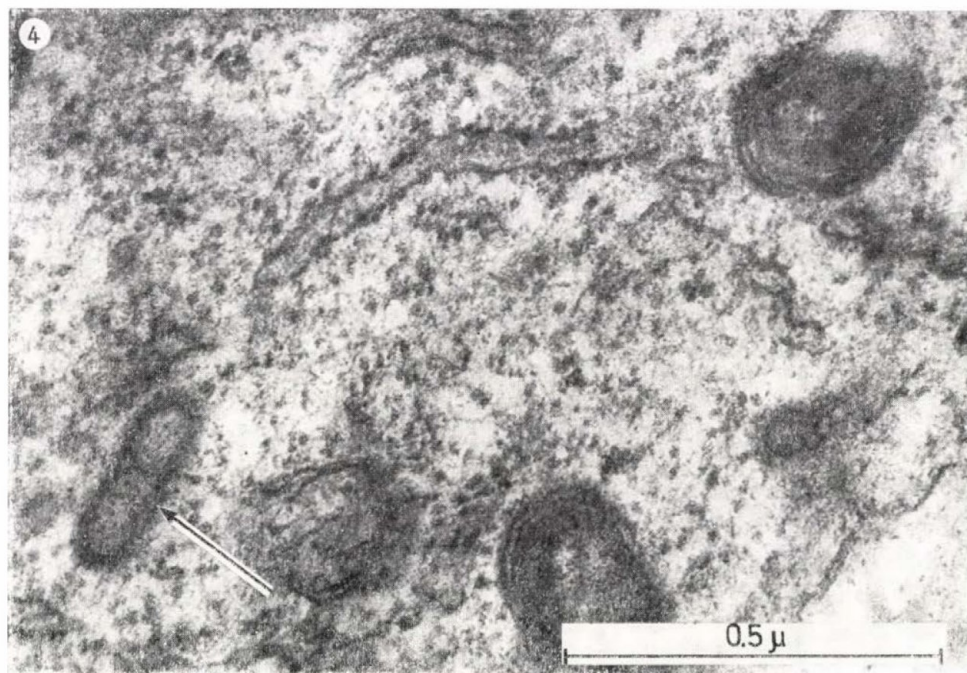


Fig. 4. The arrow points to an uncoated virion core lying free in the cytoplasm. Note the loose filamentous structure of the core. On the right of the core an unknown structural element is visible (Photo No. 9206). $\times 100,000$

150 $\text{\AA}$ thick. Among the two membranes limiting the nucleoid, the inner one is about 100 $\text{\AA}$, the outer is about 60 $\text{\AA}$ thick. The longitudinal axis of the mature nucleoid ranges from 180 to 220 $m\mu$.

Fig. 4 shows an adsorbed (at the bottom) and an engulfed virion (in the upper left corner), the latter is enclosed in a reduplicate of the cytoplasmic membrane. The arrow points to the uncoated core of a virion (see explanation to Figs 2 and 9). Besides, endoplasmic reticulum and ribosomes are to be seen.

Fig. 5 presents infected cells in which no virus particle has yet developed. In this early phase of infection chorionic cells show characteristic changes even before the appearance of "factories". The nucleus of the upper cell has two lobules. In the cytoplasm on the left of the nucleus an increased number

of mitochondria, whereas in the concavity of the nucleus partly a filamentous structure, partly a structureless substance characteristic of viral inclusion is visible. Two similar inclusions are on the right of the lower nuclear lobule. At the middle level of the picture an intercellular border is to be seen. In the lower cell, mitochondria and elements of the endoplasmic reticulum have unusually increased in number.

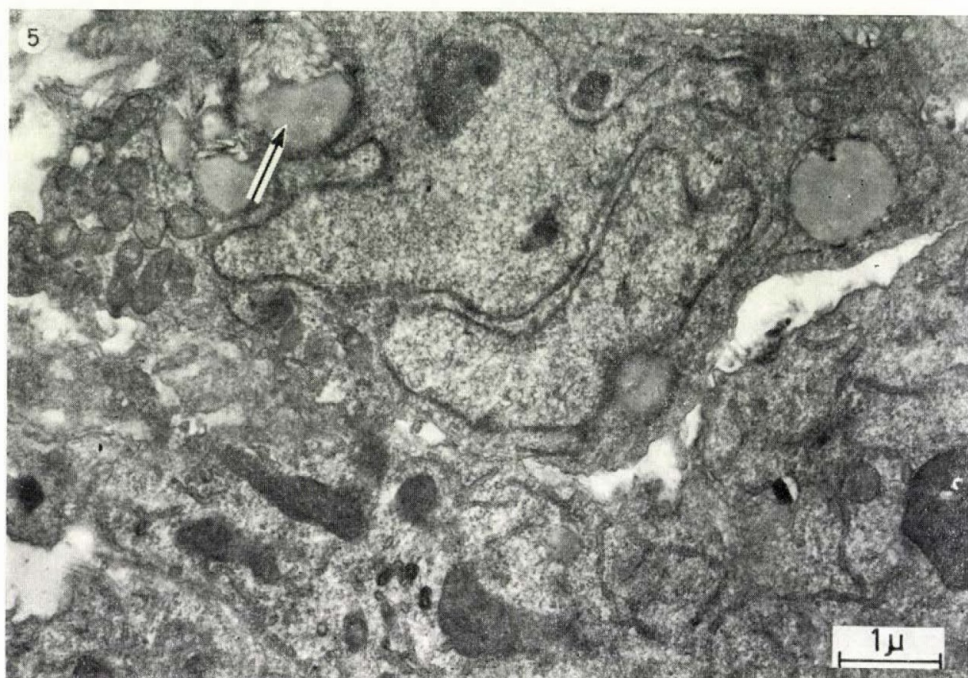
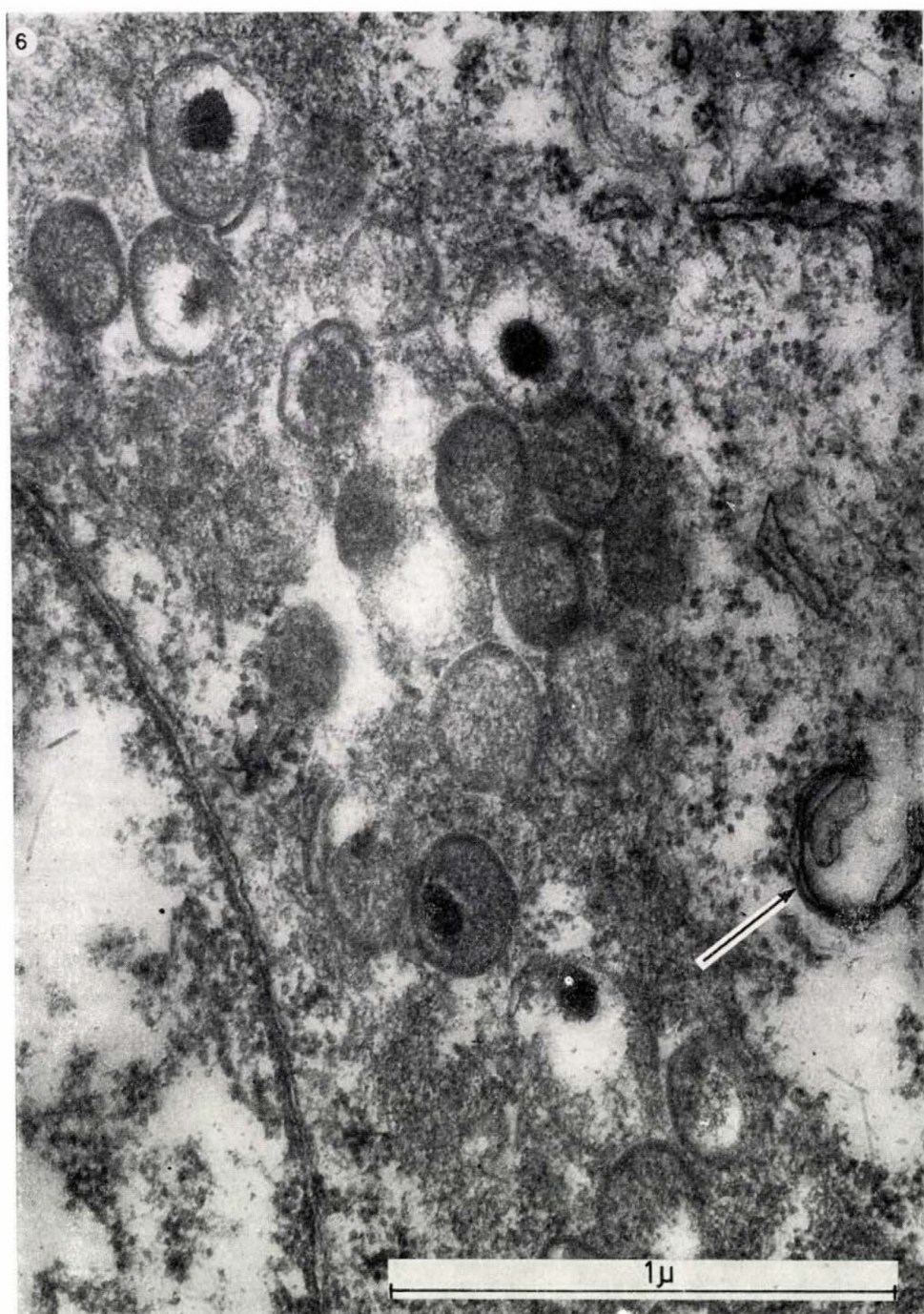


Fig. 5. An infected cell before the appearance of typical factories (Photo No. 7169). $\times 13,000$

Logarithmic phase of viral reproduction. From the 6th postinoculation hour on, the fine filamentous structure of the juxtanuclear matrix of virus factories begins to appear (v. in the left lower part of Fig. 6). It is surrounded by a rarified cytoplasm showing an accumulation of mitochondria and ribosomes (on the left in Fig. 5). From the substances of the matrix the membranes of several immature particles appear almost simultaneously. These show first an incomplete, later a complete, ovoid form. The substance in the area enclosed by the membrane is in the beginning similar to the matrix. In some particles an especially intensive, eccentric condensation, the nucleoid, appears. It is surrounded by a lighter zone. The nucleoid seems to develop independently of the condensation of the viroplasm and the development of any other virus



Figs 6—9. Logarithmic phase of reproduction

Fig. 6. The arrow points to a portion of a network of canalicles of laminar structure of unknown function (Photo No. 9224). $\times 70,000$

structure. Matrix-embedded nucleoids may occur without other viral structures and a nucleoid may occur in immature particles containing a relatively loose viroplasm.

Fig. 7 shows approximately the same as Fig. 6. In the immature formation framed and inserted at a greater magnification (Fig. 8) the filamentous, reminding of helical, structure of the nucleoid is well visible.

In Fig. 9 immature particles are shown at greater magnification. The arrow points to a formation corresponding to the one described by DALES and SIMINOVITCH [5] and DALES [7]. According to these authors this formation represents the core of the engulfed virion that has emptied its DNA content.

In our electron micrographs immature particles vary in size according to the section plane. The longitudinal axis of the largest particles ranges from 290 to 350 m μ , that of the shorter diameter from 180 to 250 m μ .

Late period of viral reproduction. Figs 10–15 show the morphology of the late period. This is characterized by the simultaneous presence of mature and immature formations in chorionic cells. In Fig. 10 immature particles are to be found near the nucleus, often in its concavity, whereas the mature virions are shifted to the apical parts of the cytoplasm. Only few of the mature virions are surrounded by endoplasmic reticulum. In Fig. 10, in accordance with literary data, there are no transient forms between immature particles and mature virions. Such particles were rare in our pictures. In Fig. 11 the virus-producing matrix is located on the left. At its lower pole there are three immature forms, while upwards from this point particles of variable maturity appear among the saccules of the Golgi apparatus. In the upper right corner of the picture some transient forms are seen; a high-power picture of these is shown in Fig. 12 where the particle in the lower left corner has a double membrane similar to that of the mature virion. The dense, ovoid, immature nucleoid has become a flattened, loose structure, but the disc-like form has not developed yet. Below the nucleoid one of the lateral bodies is already discernible.

The own membrane system of the nucleoid is also in the developmental stage. The two particles on the right appear to be at the same degree of development.

In Fig. 13 mature virions cut in different planes are visible in the cytoplasm. The cell membrane falls just out of the picture near the upper left corner. The virions are not enclosed in envelopes, though there are ribosomes and even elements of the endoplasmic reticulum to be seen.

In Fig. 14 the mature virion beside the Golgi apparatus is surrounded by a double layer originating from the nongranular endoplasmic reticulum. The later is imperfect just below the left pole of the particle. It should be noted that, although similar formations are not infrequent in these prepara-

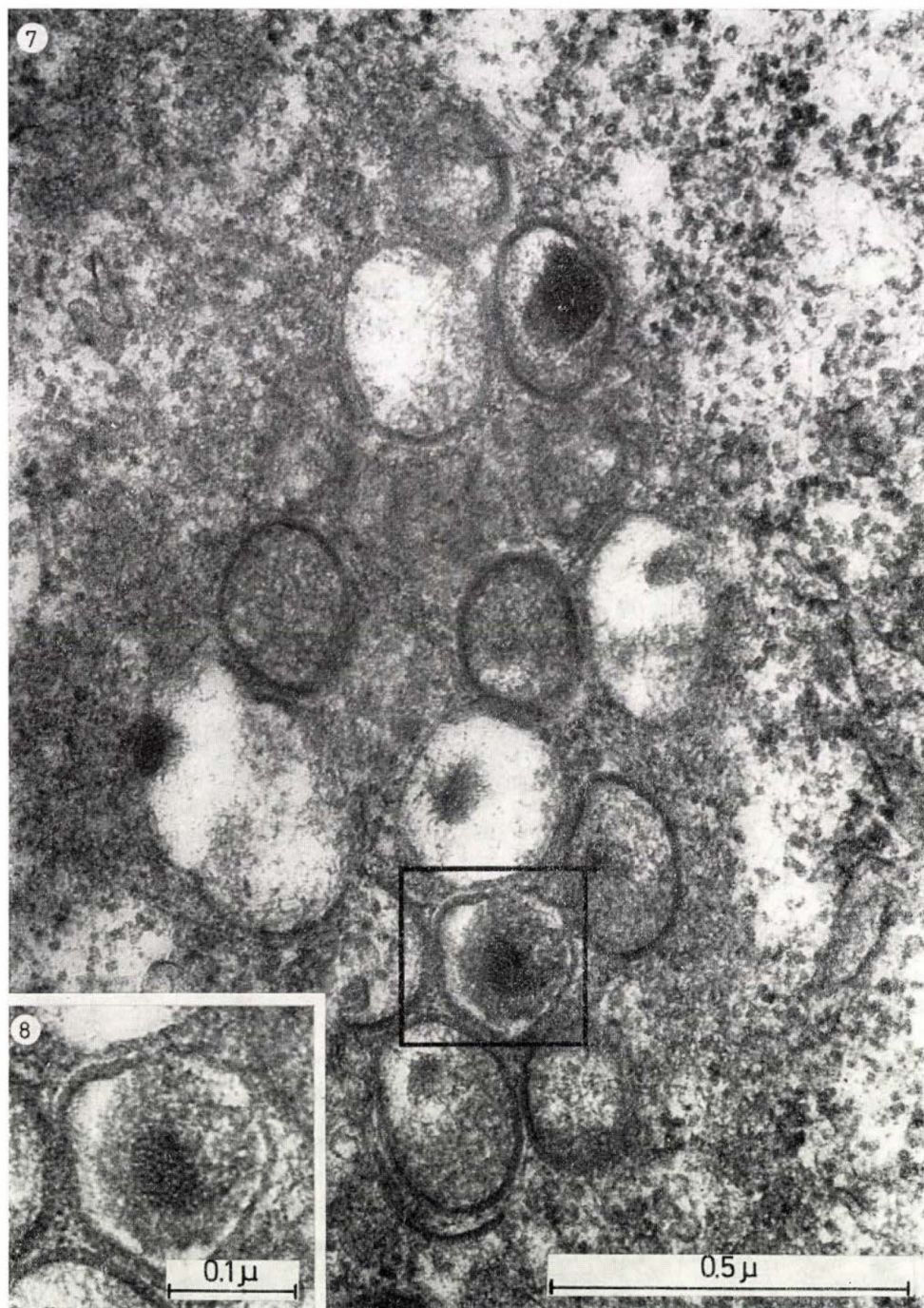


Fig. 7. Note the characteristic immature particles in the matrix. Enframed a particle with a nucleoid of filamentous structure. On both sides of the matrix, accumulation of ribosome in the cytoplasm (Photo No. 9225). $\times 100,000$

Fig. 8. Enframed part of *Fig. 7*. Note the filamentous structure perpendicular to the longitudinal axis of the particle. $\times 165,000$

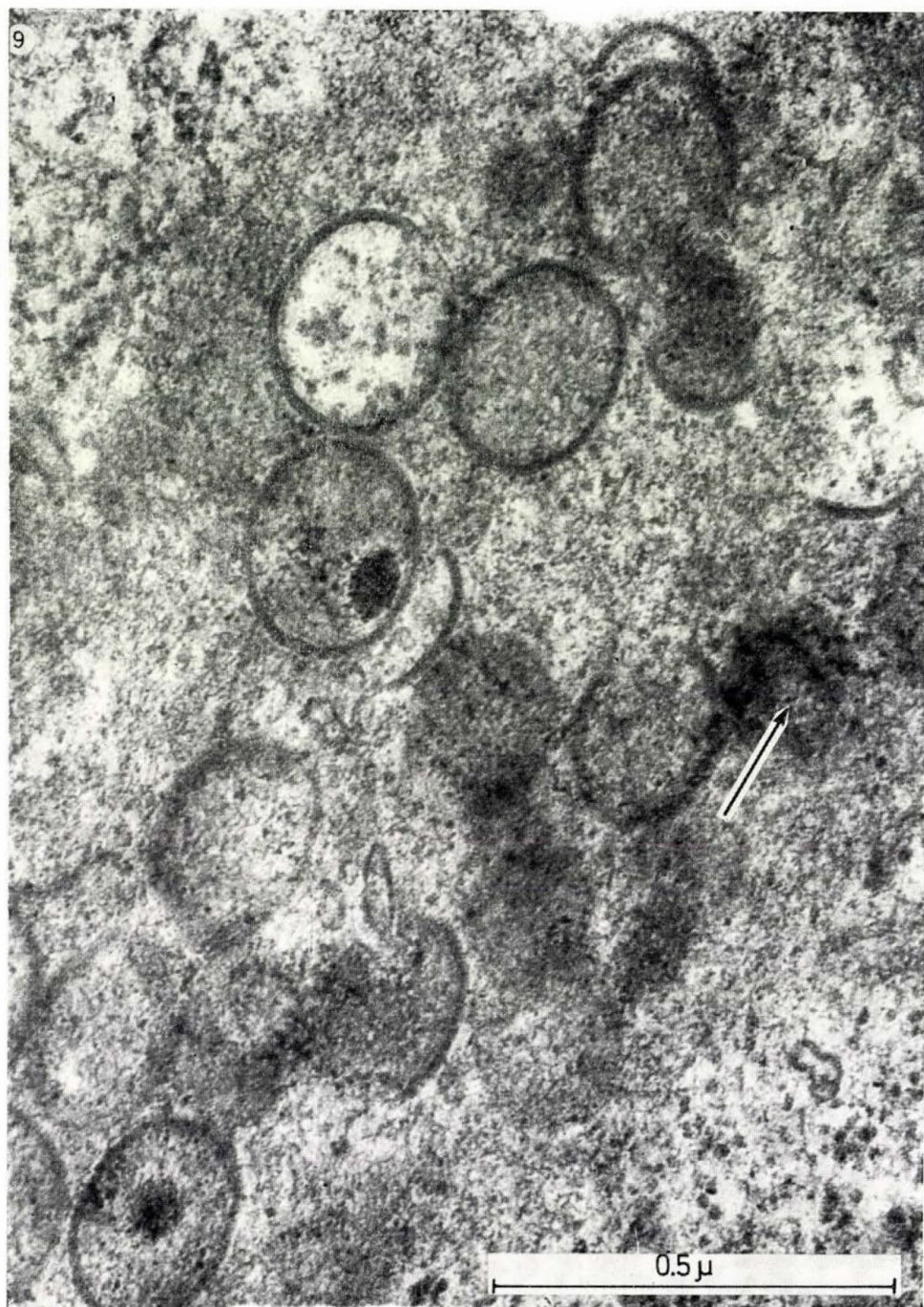
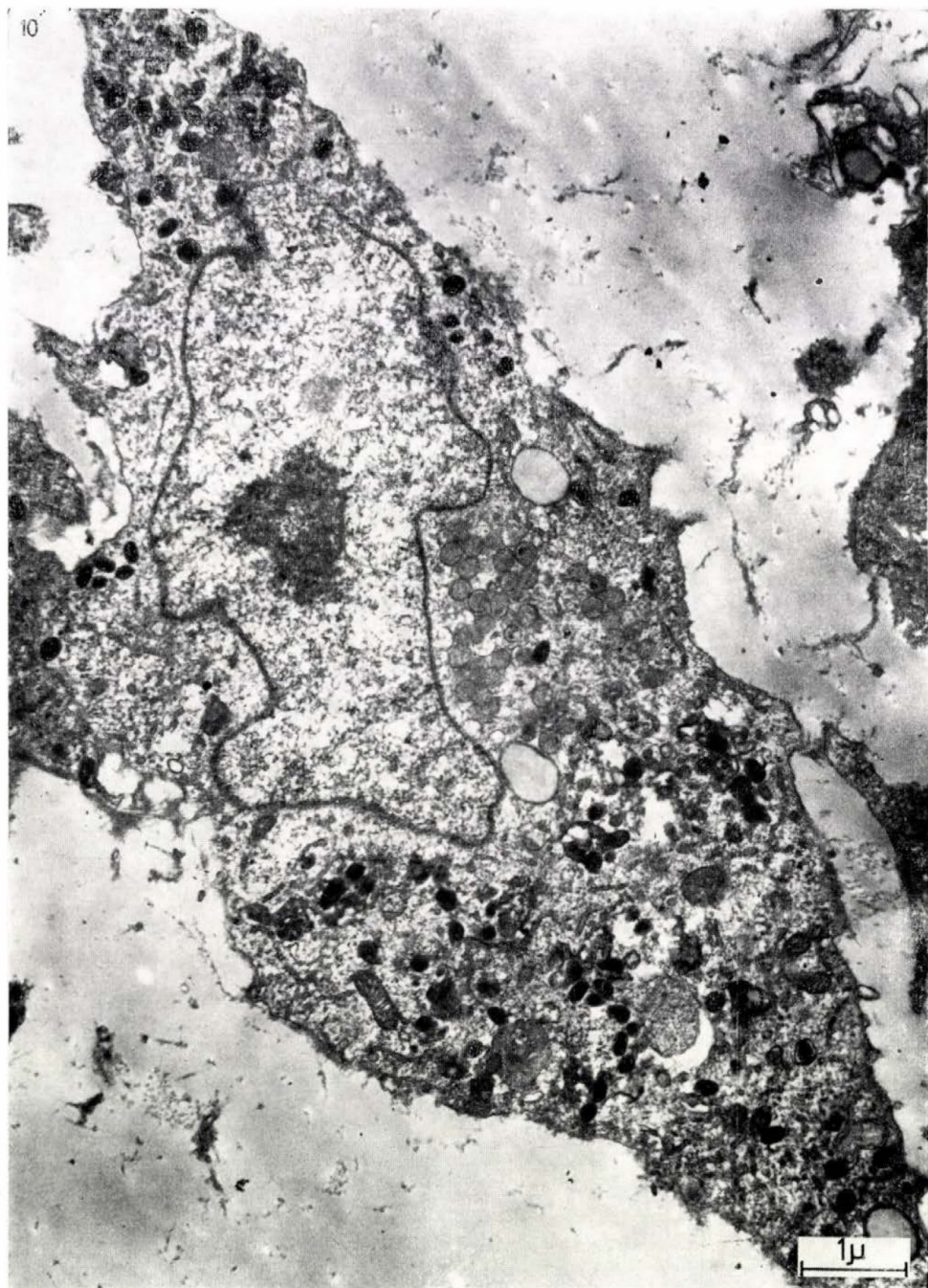


Fig. 9. Essentially the same as Fig. 8. The arrow points to an emptied virus core (Photo No. 9196). $\times 110,000$



Figs 10–15. Late stage of reproduction

Fig. 10. Immature particles in the beach of the nucleus and mature virions shifted towards the apices of cells. Numerous vacuoles in the cytoplasm (Photo No. 7066). $\times 14,000$

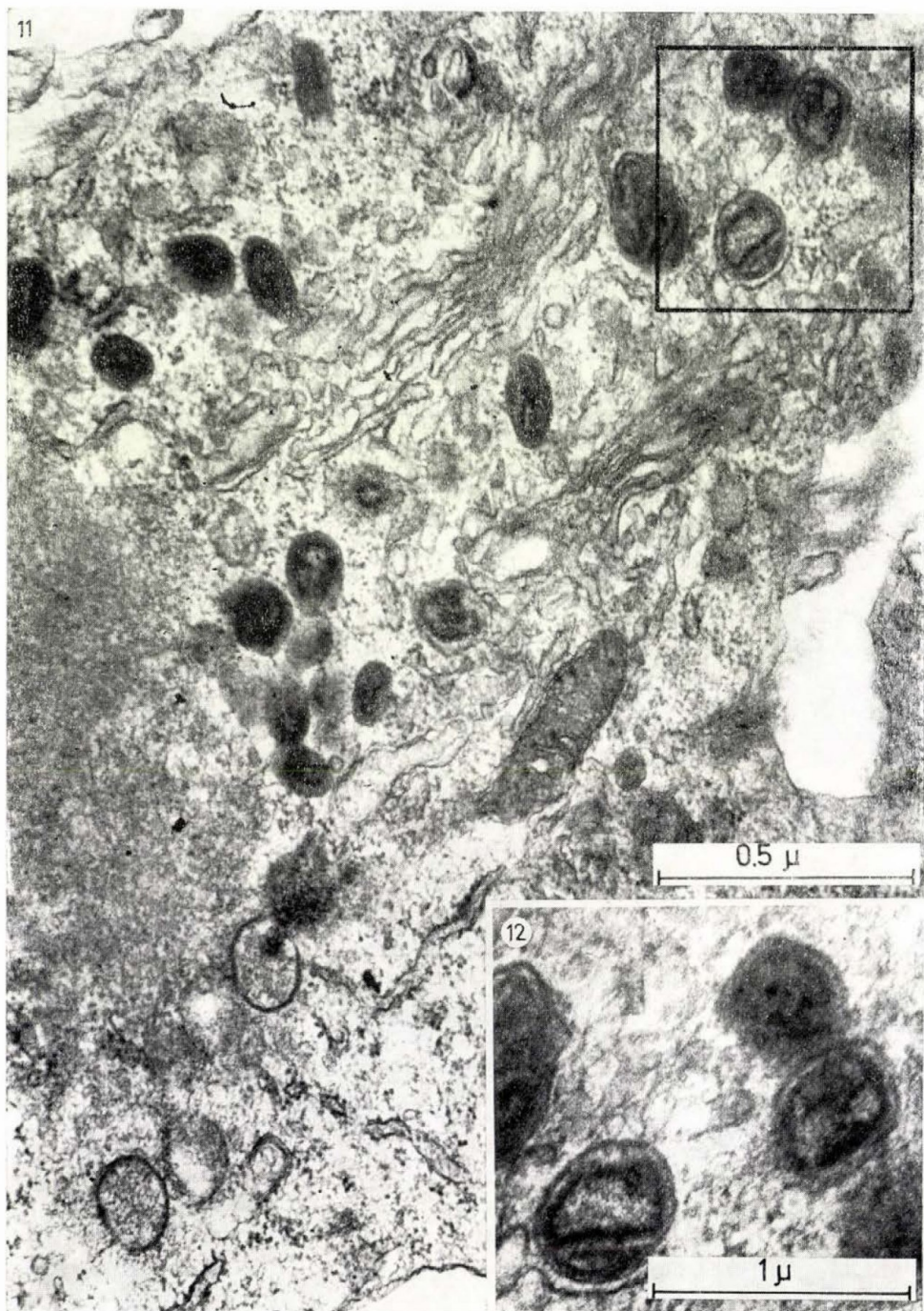


Fig. 11. Explanation in the text (Photo No. 7224). $\times 36,000$

Fig. 12. Enframed area of Fig. 11. $\times 70,000$

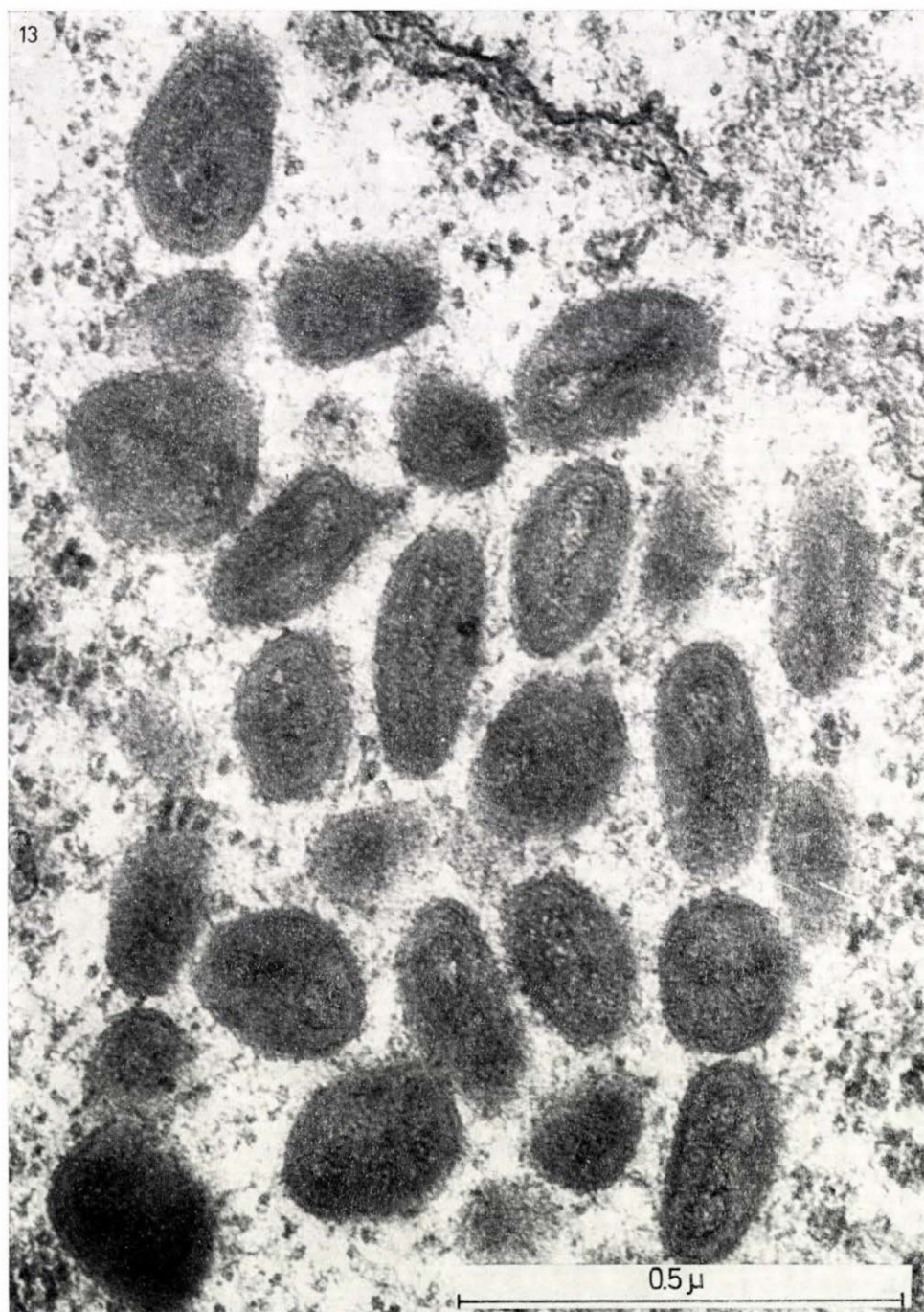


Fig. 13. Explanation in the text (Photo No. 9223). $\times 124,000$

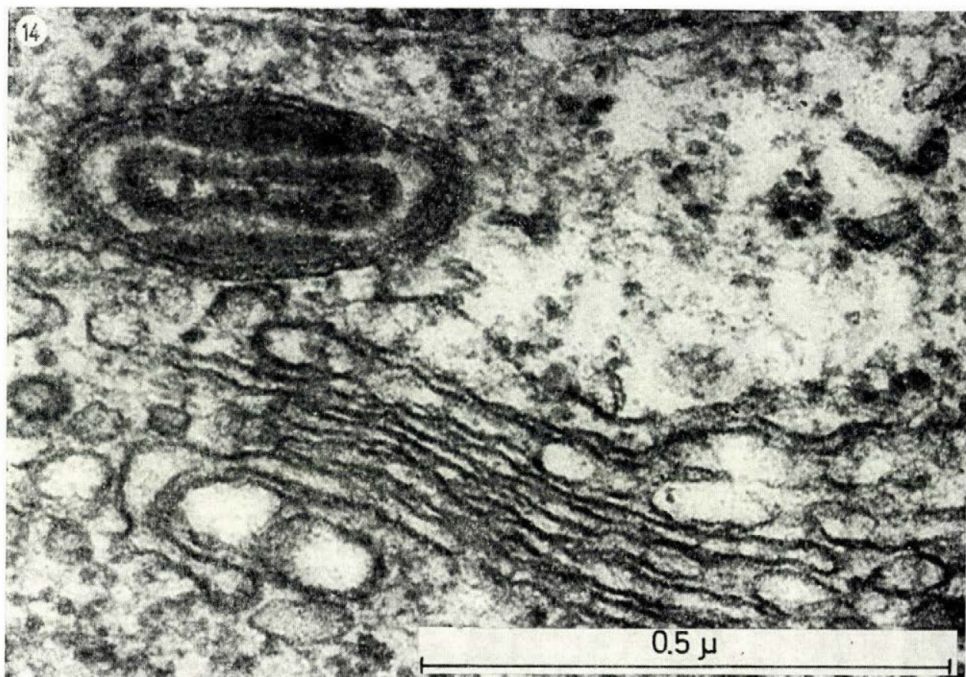


Fig. 14. Explanation in the text (Photo No. 9222). $\times 138,000$

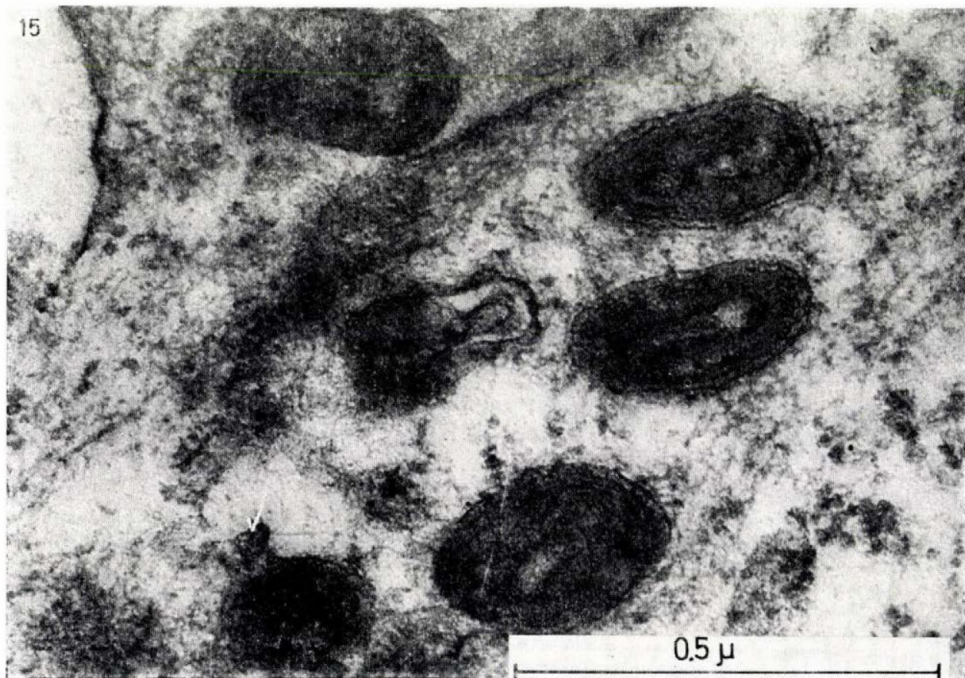


Fig. 15. Explanation in the text (Photo No. 9227). $\times 112,000$

tions, yet most of the virions lie free in the cytoplasm and particles surrounded by a vacuole are extremely rare.

The particles in Fig. 15 are similar to those in Fig. 14.

Discussion

The morphological course of the reproduction of vaccinia virus in the CAM was not revealed by the early investigations [1-4] which, owing to the undeveloped techniques, were restricted to the description of the virus-producing matrix and of mature and immature particles. There is no doubt that, in addition to the development of electron-microscopic technique in general, the tissue culture techniques and autoradiography supplied the methodical advantages that have led to the elucidation of the morphological events characterizing the adsorption, penetration and uncoating [5, 10, 11, 12] and subsequently, of those characterizing the late stages [12], *viz.*, maturation and virus release. The investigations concerning the reproduction of vaccinia virus have been reviewed by JOKLIK [19].

Although the simplicity of cell culture systems provides some advantage, the CAM system is also suitable for such investigations.

We have found the CAM preferable to monolayer cell cultures in investigating the antiviral effect of metisazone [18]. This has prompted us to attempt to find the corresponding developmental forms in the chorionic cells of the CAM. It is true that the quantitative relations cannot so exactly be regulated in this system than in cell cultures, as neither the area of the artificial air-sac nor the number of the cells to be infected is known accurately. To diminish the difficulties concerned, we used inocula greatly different in size. It has been found that the intracellular processes are well evaluable, independently of the size of the inoculum. As expected, high-multiplicity infection proved to be most convenient for the study of the early phases, but the picture of the late phases was independent of the inoculum size.

In our opinion the embryonated egg, which provides a tractable system, may yield results equivalent to those obtainable with tissue culture system. We could observe every formation that has been described for cell cultures characteristic of the adsorption, penetration and uncoating, including the core freed of viroplasm and outer membranes. In the matrix, which is different in structure from the normal cytoplasm, we could find immature single-membraned forms which are larger than the mature virion. Some of our electron-micrographs provided new data concerning the maturation of the nucleoid. Transient forms were quite exceptional. In our opinion maturation of the virion, *i.e.* the transformation of the more or less ovoid, dense or filamentous nucleoid into the characteristic, impressed, disc-like, double-

membraned nucleoid characteristic of the mature virion is a quick process as related to the intervals between samplings.

The successive infectivity titrations were insufficient to determine the stage of development in which virus particles become infectious. In this respect further studies are in progress.

As regards the cytological changes of the vaccinia-virus-infected chorionic cells, we have observed inclusion bodies, apparently of the Guarnieri type, well distinguishable from the matrix described above as well as from small cytoplasmic vacuoles. Accumulation of mitochondria and ribosomes around the virus-producing matrix was obvious. The endoplasmic reticulum showed no appreciable changes. In some of the photos we have found the network of canalicles of laminar character, already described by others as a system of unknown function.

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HALIDE AND ALKALI ION UPTAKE BY STREPTOMYCES AUREOFACIENS

By

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Summary. Comparative studies have been performed on halide and alkali ion transport in *Streptomyces aureofaciens* strains B-28 and CDSD-314.

The uptake of K^{42} is a mediated transport process leading in 40 to 60 minutes to an equilibrium characterized by a considerably higher intracellular than extracellular K^+ concentration. The transport of K^+ is inhibited by azide. The selection between K^+ and Na^+ is strongly favourable to K^+ .

There is a linear relationship between the extracellular and steady state intracellular bromide level; the latter remains lower than the former. The equilibrium is reached in less than 5 minutes and the bromide content can be washed out or exchanged in a short time. Azides increase the steady state bromide content of the cells. The results indicated that bromide uptake is a non-mediated process and the steady state concentrations are determined by the Donnan equilibrium.

When the osmolarity of the solutions is increased, both K^+ transport and steady state Na^+ or bromide content increase. No osmoregulative mechanism based on K^+ transport was revealed in *Str. aureofaciens*.

The transport properties of the two *Streptomyces* strains showed minor quantitative differences (for example, the steady state Na^+ and K^+ content was lower in strain CDSD-314 than in strain B-28). The failure of CDSD-314 to produce chlortetracycline was, however, not attributable to a mutation in halide transport.

Studies on the tetracycline formation by chlortetracycline and tetracycline producing *Str. aureofaciens* strains [4, 9—11] have indicated that incorporation of the halide occurs in a relatively late step of the biosynthetic process. It has been supposed [5] that chlortetracycline-non-producing strain CDSD-314 is deficient in the step responsible for enzymatic chlorination. Without transport experiments, however, it could not be excluded that strain CDSD-314 was in reality a chloride transport mutant.

In the last twenty years increasing attention has been paid to transport processes in bacteria. The transport of halides has been examined in detail by SCHULTZ *et al.* [18]. *Actinomycetes* have been neglected in this respect; only one report on the amino acid transport in *Str. hydrogenans* was available [14].

To examine the role of halide transport in chlortetracycline production, we compared the bromide transport in chlortetracycline-producing (B-28) and tetracycline-producing (CDSD-314) strains. In view of the very low equilibrium levels ^{82}Br was used instead of the less sensitively measurable ^{36}Cl .

In order to compare the results with the extensive data described for *Escherichia coli*, it seemed necessary to determine the most important characters of alkali cation (K^+ and Na^+) transport.

Materials and methods

Organism. The strains were described in detail by KOLLÁR and JÁRAI [1, 8]. Strain B-28 produces chlortetracycline in the presence of suitable chloride concentrations and tetracycline in the absence of chloride. Strain CDS-314 produces only tetracycline independently of the chloride content of the medium. In strain B-28 chlortetracycline production is competitively antagonized by bromide ions, in the presence of which the culture produces bromtetracycline. The production of chlortetracycline can be inhibited with 2,5-dimercapto-1,3,4-thiadiazole; in media containing this substance the strain produces tetracycline.

The experiments were performed with standardized frozen cultures prepared as follows. Spores obtained from Czapek—Dox medium were inoculated into flasks containing "inoculum medium" and shaken for 48 hours at 27°C and 220 r.p.m. The cultures were then united, supplemented with 5% glycerol, distributed at 10 ml portions and frozen at -20 to -30°C. The cultures were stored at -15°C and in each experiment 5 ml aliquots were transferred into "inoculum medium" distributed at 70 ml portions into 500 ml Erlenmeyer flasks. *Inoculum medium*: starch, 2%; corn steep liquor, 2%; $CaCO_3$, 0.5%; pH 6.8. Cultures prepared in the "inoculum medium" were used for inoculating 70 ml amounts of medium T distributed into 500 ml Erlenmeyer flasks. *Medium T*: soluble starch, 3%; filtered corn steep liquor, 0.6%; $CaCO_3$, 0.6%; NH_4NO_3 , 0.7%; pH 7.0. The cultures were shaken for 24 hours at 27°C and 220 r.p.m. then forwarded from the laboratory to the Institute of Plant Physiology where they were shaken for further 16 hours.

Uptake experiments. Thirty minutes before the experiment the cultures were centrifuged and washed twice in sterile medium G. *Medium G*: glucose, 2%; NH_4NO_3 , 0.4%; $MgSO_4$, 0.025%. The washed material was then suspended in medium G so as to contain 10 to 20 mg dry organisms per ml (the dry weight content was determined in parallel aliquots). The suspension was shaken at a constant temperature until the beginning of the experiment.

In the uptake experiments medium G or solutions of Br^- , K^+ or Na^+ in distilled water were used. In some experiments respiration inhibitors, osmotics, etc., were also added. To 20 ml solution 1 ml *Streptomyces* suspension was added (that is, when distilled water was used, the solution represented a 1 : 20 dilution of medium G). During the uptake experiments the suspensions were shaken at a constant temperature.

After the uptake period the suspension was passed through Selecta quantitative filter paper placed on a Büchner funnel. It should be mentioned that membrane filters widely used in microbiological transport experiments were unsuitable for *Str. aureofaciens* cultures because filtration through them was not sufficiently rapid. After filtration the organisms were washed twice with 10 ml solution (medium G, distilled water or salt solution according to the type of the experiment). The material from the filter paper was first transferred into small tubes, then into a test tube where it was suspended in 3 ml distilled water.

Isotopes ^{82}Br , ^{42}K and ^{24}Na were determined by the use of a Frieske—Hoepfner 49 apparatus supplied with a scintillation well counter. The data were calculated for $\mu Eq/g$ dry weight.

Some experiments were performed in order to show the degree of bromide ion incorporation into organic compounds. Twenty-four hour cultures were incubated further for 16 hours in medium T containing an "almost carrier-free" labelled bromide preparation of some hundred μC activity. The organisms were extracted with 0.01 N HCl after the usual filtration and washing. One aliquot of the extract was chromatographed by the descending technique on Schleicher—Schüll 2043/B paper moistened with Selecton B. The chloroform phase of butanol : phosphoric acid : chloroform (1 : 4 : 9) was used as a solvent. (The phosphoric acid solution contained concentrated phosphoric acid, 20 ml; trichloroacetic acid, 1 g; distilled water, 1000 ml.) On the chromatograms, in addition to the spot corresponding to inorganic bromide, a spot with a higher R_f value was detected. This component, probably identical with bromtetracycline, was responsible for about 10% of the total activity. This finding indicates that the majority of ^{82}Br was taken up as inorganic bromide.

Results

The time-course of cation and bromide ion transport was characterized by two types of curve. In the beginning the time-course for ^{42}K was linear, then the rate of transport gradually decreased until an equilibrium had been reached (Table I). Assuming that the dry weight of *Str. aureofaciens* amounts to about 20% of its wet weight, in the steady state the intracellular accumulation was several-fold of the extracellular concentration (for example about 60–80-fold at 0.5 mM). The curve for ^{24}Na transport showed hardly any rise between the 5th and 20th minute and thus, as calculated on the basis of the above assumption, the intracellular concentration of Na was lower than its extracellular concentration. As to ^{82}Br , a considerable rise was never obtained after the first determination and the calculated internal concentration reached only 10 to 25% of the extracellular concentration.

Table I

Time-course of K^+ transport and Na^+ and Br^- content in *Streptomyces aureofaciens*

(The data are expressed in $\mu\text{Eq/g}$ dry weight; ion concentration in nutrient solution, 0.5 mEq per litre; the microorganism was washed in distilled water)

Experiment	Br^-		K^+		Na^+
	23	24	22	26	25
<i>Strain B-28</i>					
5 min.	0.14	0.012	11.3	7.6	0.18
10 min.	0.18	0.013	33.5	28.0	0.37
20 min.	0.21	0.013	73.8	56.8	0.56
40 min.	—	—	—	120.4	—
60 min.	—	—	—	125.2	—
<i>Strain CDS-314</i>					
5 min.	0.13	0.009	50.1	12.6	0.94
10 min.	0.13	0.009	82.8	34.2	1.32
20 min.	0.17	0.010	102.1	68.4	1.52
40 min.	—	—	—	133.0	—
60 min.	—	—	—	161.2	—

Note. In Experiment 24 the concentration of Br^- was 0.1 mEq/l.

If the intracellular ^{42}K , ^{24}Na and ^{82}Br contents are compared to the respective external concentrations, it is evident that K^+ differs in behaviour from Br^- and Na^+ . The activity of these two ions is directly proportional

to the extracellular concentration; in contrast, the activity of K^+ is independent of the concentration (Fig. 1).

As shown in washing and exchange experiments the K^+ and Br^- content of *Str. aureofaciens* could be exchanged (Table II). The rate of exchange (+ washing out) for these ions differed considerably; while the Br^- content was replaced almost totally in 5 minutes, at the same time only about 40% of the K^+ content was exchanged.

Table II

Exchange of K^+ and Br^- content in Streptomyces aureofaciens

(The data are expressed as percentage of the corresponding control; ion concentration in nutrient solution, 0.5 mEq/litre; time of uptake, 20 minutes)

Experiment	K per cent	Br per cent
	21	23
<i>Strain B-28</i>		
Distilled water, 20 seconds	100	100
2.5 mEq/l $CaSO_4$, 20 seconds	95	78
10 mEq/l KCl, 20 seconds	72	36
10 mEq/l KCl, 5 minutes	58	8
<i>Strain CDS-314</i>		
Distilled water, 20 seconds	100	100
2.5 mEq/l $CaSO_4$, 20 seconds	95	109
10 mEq/l KCl, 20 seconds	88	88
10 mEq/l KCl, 5 minutes	60	22

From the above data it may be concluded that K^+ uptake is an active mediated transport process. At the same time these data do not prove the mediated transport of Na^+ and Br^- . The time-course and the rapid exchange of Br^- indicate that the cells are permeable to these ions and thus the low intracellular concentration is due to a Donnan equilibrium and not to impermeability.

Measurements lasting longer than 5 minutes reflected the Na^+ and Br^- steady state reservoir in the cell. As to K^+ , short-term measurements indicated the transport rate, long-term ones the steady state reservoir. As K^+ transport was measured only by radioactivity counting and not by determining the K content chemically, no conclusions can be drawn as to whether the data reflect a net accumulation or an exchange influx in the cell possessing a steady state K reservoir. In view of the fact that at high ammonium ion content

medium G contains K^+ only as a contaminant and at very low concentration, a net accumulation seems more probable.

The rate of K transport was strongly decreased by 0.1 mM azide (Table III). The inhibition was more definite in undiluted than in diluted

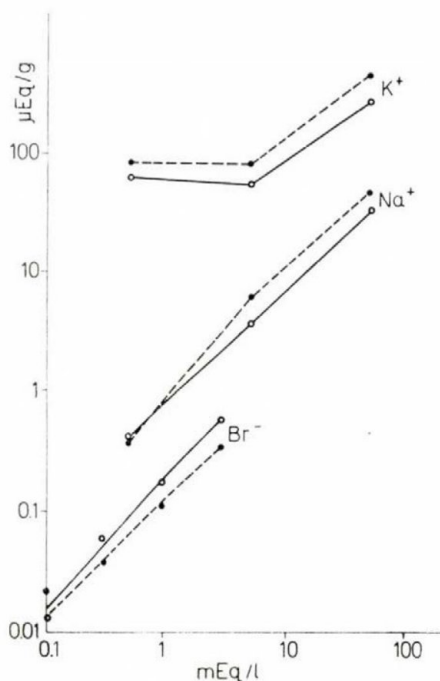


Fig. 1. Concentration curves for K^+ transport and Na^+ and Br^- content in *Streptomyces aureofaciens*; $\mu\text{Eq/g}$ dry weight; uptake in 20 minutes from nutrient solution. The micro-organism was washed in distilled water (K^+ and Na^+) or medium G (Br^-). Solid line: strain B-28; dashed line: strain CDS-314

medium G. The addition of azide considerably increased the steady state bromide content. The stimulating effect was especially well observed in undiluted medium G. Our earlier experiments also indicated that the azide had no inhibitory effect in the case of Br^- . Thus K^+ transport must be an active process. The stimulating effect of azide on steady state bromide content might be explained by supposing that the agent inhibits an active efflux step of a transport process; this interpretation is, however, in contrast with other data. It may be supposed that azide causes an alteration in the bioelectric potential between the cells and the environment and thus influences the anion's distribution. The size of *Streptomyces* cells is too small to allow bioelectric measurements, so that the hypothesis cannot be proved.

Our experimental data for *Str. aureofaciens* were similar to results obtained for the cation and halide transport in *E. coli*. In this respect the only marked difference between the two strains was in the effect of the medium's osmolarity.

Table III

*The effect of 10^{-4} M azide on K^+ and Br^- transport in *Streptomyces aureofaciens**

(K uptake from 10 mM KBr solution, 10 minutes. Br uptake from 0.1 mM KBr solution, 10 minutes. The microorganism was washed in distilled water [K^+] or nutrient solution [Br^-]. The data are expressed in $\mu\text{Eq/g}$ dry weight or percentage of the corresponding control)

Experiment	Br^-		K^+	
	27		26	
	$\mu\text{Eq/g}$	per cent	$\mu\text{Eq/g}$	per cent
<i>Strain B-28</i>				
Nutrient solution 1 : 20	0.052		54.4	
Nutrient solution 1 : 20 — azide .	0.042	80	24.8	45
Nutrient solution	0.0072		32.8	
Nutrient solution — azide	0.054	750	4.1	12
<i>Strain CDS-314</i>				
Nutrient solution 1 : 20	0.048		69.4	
Nutrient solution 1 : 20 — azide .	0.120	250	31.8	45
Nutrient solution	0.024		25.8	
Nutrient solution — azide	0.066	275	5.08	19

As shown in Table III (and as revealed in unpublished experiments) K^+ transport is slower and the steady state bromide content is lower in undiluted than in diluted medium. This finding may be explained by differences in the osmolarity of the media. In order to confirm this consideration, the effect of polyethyleneglycol and sucrose solutions of known osmolarity was examined (Table IV). The effect was similar for all three ions in that the transport rates and equilibrium levels decreased. The decrease in the latter could not be responsible for the finding, because it was higher than that expected on the basis of the osmotic loss of water (decrease in volume). K transport in *E. coli* plays an osmoregulative function [3] and in a similar experiment the two ions would therefore behave in an opposite manner: the K^+ content would increase parallel with osmolarity.

Table IV

*The effect of osmotics on K^+ , Na^+ and Br^- transport in *Streptomyces aureofaciens**

(Ion concentration, 0.5 mEq/litre; time of incubation, 20 minutes.
The microorganism was washed in distilled water. The data are expressed as percentage of the corresponding control. Nutrient solution diluted 1 : 20)

Experiment	K^+	Na^+	Br^-
	22	25	24
<i>Strain B-28</i>			
Polyethyleneglycol "1000"	44	28	28
Sucrose	64	84	77
<i>Strain CDS-314</i>			
Polyethyleneglycol "1000"	61	38	45
Sucrose	87	113	31

Note. Osmolarity of both substances, 4.2 atm. Polymerization of polyethyleneglycol, 19–20 (British Drug Houses).

Discussion

As transport processes in *Actinomycetes* are practically unknown, it seems advisable to compare our data with those for *E. coli*.

In *E. coli* two different K transport processes can be distinguished. 1. K–K exchange in cells rich in K. 2. Net K uptake by cells poor in K [15–17]. The concentration of equilibrium level K in the cells ranges between 200 and 240 mM. This K content is in constant exchange with extracellular K ions. In short-term experiments the rate of exchange is decreased by DNP without a change in the equilibrium concentration. In the examined concentration ranges (0.06 to 13.8 mM) the rate of exchange was independent of the concentration, thus the K_m of the mediated process is considerably lower than the concentrations used.

When *E. coli* cells poor in K content are transferred into K-containing medium, a net K^+ uptake occurs. The rate of this process depends on the concentration according to the saturation curve; K_m is 4.5 mM. The process is sensitive to respiratory enzyme inhibitors such as DNP or mono-iodoacetate. The net K^+ uptake is accompanied by a simultaneous Na^+ and H^+ extrusion and is not associated with an anion accumulation in the medium. A similar net K^+ transport was described in *Streptococcus faecalis* [21].

As regards their chloride content, the bacteria can be divided into two groups. In the first group the intracellular chloride concentration is markedly

lower than the extracellular one (*E. coli*, *Staphylococcus aureus*, *Salmonella oranienburg*, etc.); in the second group the intracellular chloride content approaches or reaches the extracellular concentration (one marine *Pseudomonas* species, various halophilic *Sarcina* and *Halobacterium* species [2, 13, 18, 19]).

SCHULTZ *et al.* [18] demonstrated that in *E. coli* the intracellular chloride content was about 33% of the concentration in the medium and was in the examined range directly proportional to the extracellular concentration. The chloride content showed no change between 30 and 90 minutes incubation.

The above data indicate that in *E. coli* K^+ transport is an active, mediated process and that chloride transport is a passive, probably non-mediated process. The association between K^+ — Cl^- exchange and net uptake has not been sufficiently elucidated. As far as it may be concluded from our observations, the transport processes in *Str. aureofaciens* are similar to those described in *E. coli*.

In *E. coli*, net K^+ transport is an osmoregulative process; when transferred into hypertonic medium, the cells which have reached a steady state K^+ reservoir, accumulate additional K^+ proportionally to the increase in osmolarity [3]. This mediated kinetics process ($K_m = 1\text{ mM}$) involves a K^+ — H^+ exchange similarly to the net K^+ uptake by cells poor in K^+ . The exact mechanism of the process is unknown and the opinion of EPSTEIN and SCHULTZ [3] that mediated K^+ transport is activated by the alteration of the membrane tension must be considered a hypothesis for the time being.

Our data indicate that K^+ transport in *Str. aureofaciens* is not an osmoregulative process. If osmoregulation occurs in the cells, it takes place through some other mechanism, for example through the production of osmotically active components, like in some other microorganisms [6, 7, 12]. The decrease of the K^+ transport rate in *Streptomyces* in solutions of higher osmolarity might be associated with the need for transport energy, in analogy with the observations that in hypertonic solutions the respiration of *Saccharomyces* is inhibited [12] and the ATP content of higher plants decreases [20]. These considerations are, of course, not applicable to bromide ions. If it is assumed that the steady state bromide content in *Streptomyces* is determined by a Donnan-type distribution, it may be concluded that this is the very reason why the bromide content decreases in solutions of high osmolarity where non-diffusible polymers influencing the Donnan equilibrium are partly decomposed.

In the examined transport properties strains B-28 and CDS-314 showed numerous quantitative differences; for example, strain CDS-314 reached a somewhat higher K and Na equilibrium value than did strain B-28 (Fig. 1 and Table I) and the bromide content was lower in CDS-314 than in B-28. The cause of this is not known; it should, however, be mentioned that

the difference in cation and anion content tends to be divergent and thus the lower halide content may somehow be associated with K transport. It should be emphasized that the small quantitative differences observed do not allow to conclude that the loss of chlortetracycline production in CDS-314 is a result of a defective chloride transport. It is therefore probable that the original hypothesis [5] holds true and mutant CDS-314 had lost the enzymatic chlorination step of chlortetracycline biosynthesis.

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DEVELOPMENT OF IMMUNOLOGICAL MEMORY IN MICE TO PHOSPHORYLASE ANTIGEN

I. THE EFFECT OF DOSE AND INTERVAL OF ANTIGENIC STIMULI

By

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Summary. The dose-dependence of the primary and secondary immune response in mice to phosphorylase antigen and the effect on the plasma antibody level of the length of the interval between two subliminal doses of antigen has been examined. The "primary" and "secondary" enzyme-neutralizing antibodies proved to be of the 7S type and were resistant to mercaptoethanol. Doses of 0.01 to 1 mg induced a pure immunological memory without detectable primary antibody formation; 1 to 5 mg doses gave rise to a small degree of primary antibody production. It was calculated that the threshold antigen dose (1 mg) of the primary immune response — taking 400 minutes half life time for a basis — decreased in 46 to 60 hours to the threshold value of the secondary immune response (2 to 8 μ g). When two antigen doses (each under the threshold value *per se*) were given at 1, 2, 3, 4 and 5 day intervals, optimal results were obtained if intervals of 2 days had elapsed between the injections. It has been concluded that the early development of immunological memory (X—Y transformation) falls between 2 and 2.5 days.

The further time-course of immunological memory was examined by giving at various intervals two antigenic stimuli, which were subliminal in themselves (700 and 250 μ g); administration in 2 to 8 day intervals resulted in an antibody level similar to the maximum primary reaction. In the case of 8 to 17 day intervals the antibody titre rose exponentially; and with intervals of 20 to 65 days a steady high antibody level developed. The increase in immunological memory is explained by the proliferation of memory cells.

A secondary immune response to an antigenic stimulus is termed immunological memory. It has long been recognized that the degree of secondary response is associated with the interval between the primary and secondary stimulus. Systematic studies on this problem have dealt with antigens of bacterial origin and with corpuscular antigens [4, 12, 20].

As the course of immunobiological processes following the antigenic stimulus is considerably influenced by the special features of the antigen, in the present study we examined the kinetics of the development of immunological memory by the use of a tissue antigen. As the secondary immune response depends not only on the interval of antigenic stimuli but also on the dose of the antigen, the effect of this factor and the elimination of the antigen from the blood were also examined.

Materials and methods

Preparation of the antigen. Phosphorylase-b was prepared from the pectoral muscle of roosters. The muscle was stored at -20°C . After thawing it was ground and extracted three times at 20 to 25°C with equal volumes of 0.3% NaCl and 1 mM EDTA (ethylene-

diaminetetra-acetate). At the second and third extraction the pH was adjusted to neutral (6.8–7.0) with 0.33 *N* NaOH. The extracts were united, adjusted to pH 6.1, left to stand at 4°C overnight then centrifuged. The supernatant was adjusted with 0.33 *N* NaOH to pH 7.2, then heated at 50°C for 20 minutes, cooled to room temperature and centrifuged. Subsequently a titrated amount (mostly 1/10 volume) of 0.3% Rivanol (6,9 diamino-2 ethoxyacridine lactate) was added under stirring and the mixture was centrifuged again. The yellow precipitate was homogenized in 0.05 *M*, pH 6.7 EDTA (1/40 volume of the original extract). After heating at 50°C for 10 minutes, activated charcoal was added to the homogenate until the yellow colour disappeared. After centrifugation the supernatant contained the concentrated and purified phosphorylase. The preparation was further purified by adjusting the pH to 6.1 with 1/10 volume of 0.05 *M*, pH 5.0 EDTA and refrigerating at 6°C overnight. The white precipitate was centrifuged at 6°C and redissolved in 1 *mM* 2-mercaptoethanol solution at 30°C; the pH was adjusted to 6.7–7.0 with Tris (tris hydroxymethyl aminomethane) buffer. Immuno-electrophoresis with the homologous rabbit hyperimmune serum showed one component in the preparation. With OUCHTERLONY's modified technique [22] using rabbit hyperimmune serum prepared with heated (50°C) muscle extract the presence of two other components was demonstrated. The specific activity of the preparation was 2.3 mg P_i /min/mg protein as estimated under conditions described below. The phosphorylase solution, which contained 3 to 7% protein, was distributed into ampoules and freeze dried. After rehydration 70 to 80% of the original specific activity was demonstrated. For immunization the preparation was dissolved in physiological saline pH 7.0.

Determination of phosphorylase and antiphosphorylase activity. The method described for rabbit phosphorylase-antiphosphorylase [23] was somewhat modified. To 0.3 ml diluted plasma 0.3 ml appropriately diluted phosphorylase was added. The mixture was preincubated at 30°C for 30 minutes then 0.2 ml substrate consisting of 4% glucose-1-P dipotassium salt (Reanal), 4% rabbit liver glycogen (BDH) 6 *mM* adenosine-5'-P, pH 6.7 (Reanal) was added. After incubation at 30°C generally for 60 minutes the enzyme reaction was stopped with 3.2 ml acidic tungstate solution (0.25% $Na_2WO_4 \cdot 2H_2O$ and 1.5% acetic acid, used after standing for one day). Then 2 ml modified reagent (5% ferrous sulphate and 1% ammonium molybdate in 1.75 *N* H_2SO_4) were added to 3 ml centrifuged solution and the absorbance was measured in a Unicam SP-800 spectrophotometer at 660 $m\mu$. In the standard test the phosphorylase was added to 0.3 ml 0.1 *M*, pH 6.7 EDTA; the same solution was used for diluting plasma samples. The solution used for the dilution of phosphorylase contained 0.01 *M* EDTA, 0.05% bovine serum albumin and 4 *mM* 2-mercaptoethanol, pH 6.7. Antiphosphorylase units were calculated as described previously [23] and activity was expressed as μg P/0.1 ml blood/10 minutes. Antiphosphorylase activity was regarded proportional to the amount of antibodies if the relative inhibition was between 20 and 70%. In this range the relationship between the inhibition of activity and the amount of antibodies was linear. In order to obtain a better comparability of the results, a hyperimmune rabbit antiphosphorylase serum was used as a standard.

Animals. Randomly bred BALB/c mice weighing 24 to 26 g were used. The animals were fed a standard mouse diet and received water ad libitum.

Blood samples of 0.05 ml were taken from the caudal vein by means of a Hagedorn pipette into 0.4 ml 0.1 *M* EDTA solution, pH 6.7. Samples taken from one group of animals were pooled and centrifuged. The plasma dilutions were inactivated at 56°C for 30 minutes. Inactivation was necessary not only for destroying the complement, but also for eliminating the effect of an enzyme (probably amylase) disturbing the estimation of phosphorylase activity. Antiphosphorylase determinations were performed with the above plasma-solution or its dilutions.

Results

The rooster muscle phosphorylase was weakly antigenic in mice. Doses of 1 to 5 mg produced small amounts of neutralizing antibody exceeding at most 3-fold the normal plasma level of "natural antibodies". Doses smaller than 1 mg induced no detectable primary antibody production. The antibodies appeared in the blood on the 8th day after the first injection and reached

a maximum level on the 12th day. The secondary immune response developing as a result of a repeated antigen dose given 3 to 4 weeks after the first injection was characterized by a higher antibody level and a shorter latency period (3 days). The antibody titres attained the maximum by the 5th to 9th day, remained at high levels for a few days then began to fall.

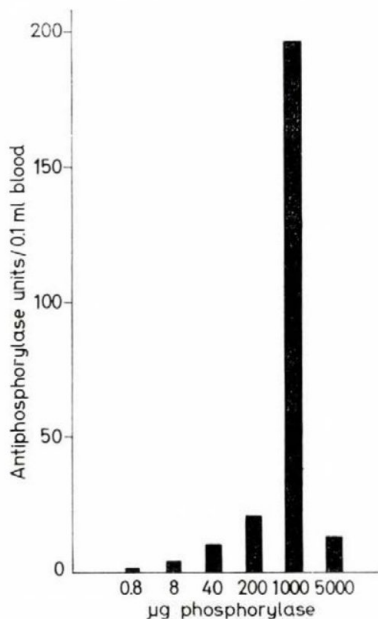


Fig. 1. Secondary immune response to various doses of primary antigenic stimuli. Each animal was given 250 μ g phosphorylase intraperitoneally 3 weeks after the primary injection. Each column shows the antiphosphorylase level in pooled blood samples of 6–7 mice 10 days after the secondary stimulus

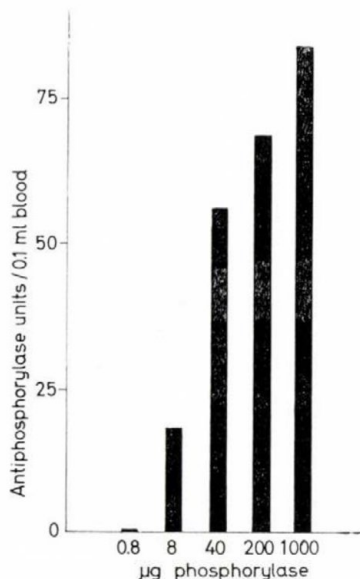


Fig. 2. Secondary immune response to various doses of secondary antigenic stimuli. Each animal was given 700 μ g phosphorylase intraperitoneally 3 weeks before the secondary injection. Each column shows the antiphosphorylase level in pooled blood samples of 6–7 mice 10 days after the secondary stimulus

Fig. 1 shows the size of the secondary immune response to various doses of primary antigenic stimuli. The antigen doses of 0.8 μ g were ineffective; 1 mg antigen exerted an optimal effect and 5 mg doses caused an immunological inhibition of the secondary immune response.

The relationship between secondary antigenic stimuli and immune response is presented in Fig. 2. The threshold dose necessary for inducing a pure immunological memory and the dose producing a secondary immune response were similar in order of magnitude (0.8 to 8 μ g). In contrast, the threshold dose giving rise to primary antibody production was 100 to 500-fold higher (1 mg). Accordingly, in our model a pure immunological memory

without antibody production (priming) could be effected with 0.01 to 1 mg antigen. It has been proved that in antibody production at least two qualitatively different stages are involved. When two sub-threshold antigen doses, either of which is insufficient to induce primary antibody production, are given, the two stages take place separately at different times. In contrast, in primary antibody production the two stages ensue consecutively. Both stages are induced by antigenic stimuli. The second stage (productive phase [49]

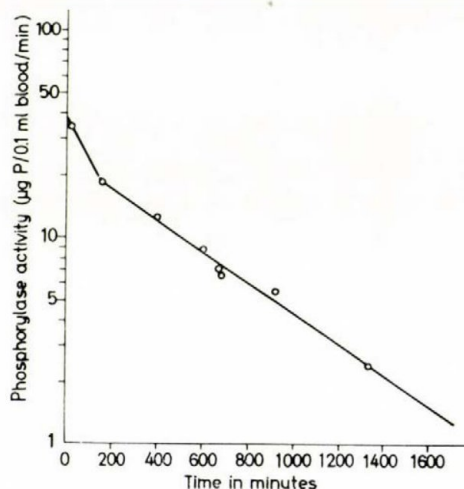


Fig. 3. Elimination of phosphorylase from mouse plasma. After injecting 1 mg phosphorylase intravenously the animals were bled at various intervals, and phosphorylase activity was estimated in the plasma dilutions. Each point represents the activity of the pooled plasma of 6 mice

or inductive phase [9]) is qualitatively identical with, or at least similar to, the process occurring in the course of a secondary immune response [50]. Accordingly, the threshold dose of antigen in the second stage of primary antibody production corresponds to the threshold dose of the secondary immune response. If the half-life of the antigen is known, the time needed for the decrease of the threshold dose antigen to the threshold value of the secondary immune response may be estimated. This value would mean the time elapsing until the second stage begins, that is, the development of memory cells.

In view of the above considerations the half-life of injected phosphorylase was determined by estimating plasma phosphorylase activity (Fig. 3). As after the injection of 1 mg antigen the half-life was 400 minutes, 46 to 60 hours later 2 to 8 µg active phosphorylase must have remained in the organism, just the amount corresponds to the threshold dose necessary for the induction of a secondary immune response. This computation indicates that the early

development of immunological memory requires approximately 46 to 60 hours.

As active phosphorylase is rapidly eliminated from the circulation — and indirect data indicate that it changes into a non-immunogenic form with the same rapidity — our model is suitable for an empirical determination of the early development of immunological memory *in vivo*. When two subliminal antigen doses were given at various intervals, the 2-day interval

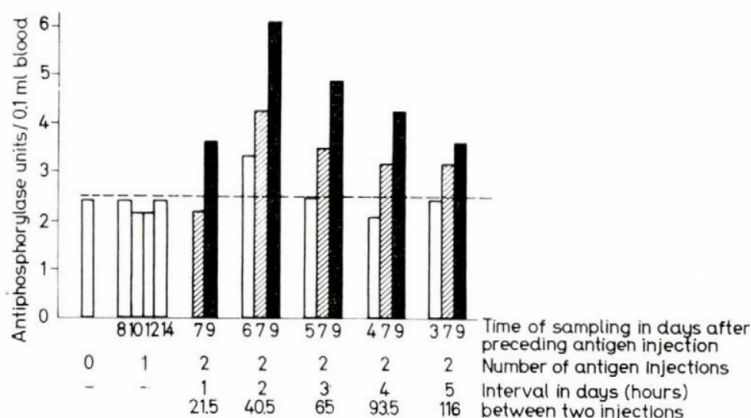


Fig. 4. Effect of the interval of two subliminal antigen doses on the antibody level. Six out of 7 groups containing 7 animals each were injected intraperitoneally with 700 μ g phosphorylase. Five groups of mice were given at different intervals 250 μ g phosphorylase as a secondary stimulus. The columns show the antiphosphorylase levels obtained in the pooled blood samples for each group. Values for each group of mice are represented by neighbouring columns

proved to be optimal. In other words, antibody production on the 7th day after the second injection occurred only if the two subliminal doses had been administered at 2 day or longer intervals (Fig. 4). This finding is in good agreement with the above estimation.

In a subsequent series of experiments the time-course of the further development of immunological memory has been studied. Fig. 5 shows the effect of two subliminal doses on the immune response. The curve illustrates that secondary stimuli applied on the 2nd, 4th, 6th and 8th day induced antibody levels similar in order of magnitude to those induced in the maximum primary reaction. Between the 8th and 17th day an exponential rise, after the 20th day a stationary phase with slowly decreasing tendency was observed.

Antisera obtained 12 days after the primary and 10 days after the secondary antigenic stimuli (2nd antigen injection between the 20th and 30th days) were subjected to sucrose gradient ultracentrifugation and 2-mercaptoethanol sensitivity testing (0.1 M, 60 minutes at 30°C). The experiments indicated that the neutralizing antibodies belonged to 7S type and were resistant to mercaptoethanol.

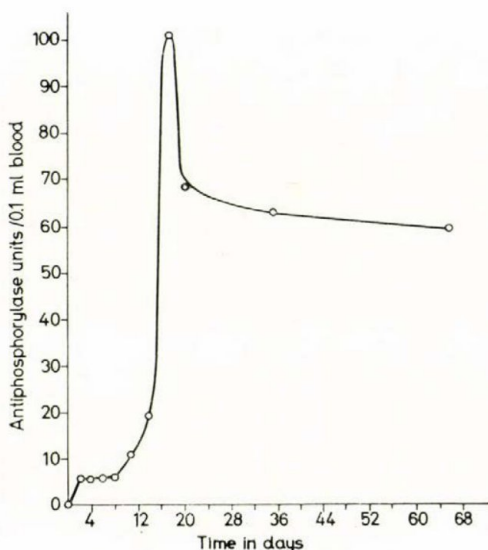


Fig. 5. Effect of the interval of two subliminal antigen doses on the antibody level. The animals were injected with 700 μ g phosphorylase intraperitoneally. The secondary antigenic stimulus (250 μ g) was applied between the 2nd and 65th day. Each point represents the antiphosphorylase level in the pooled plasma of 9–10 mice, 10 days after the secondary injection

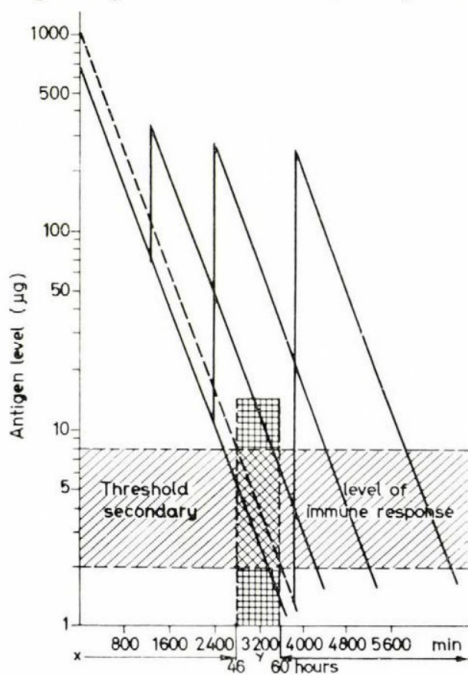


Fig. 6. Schematic representation of the relationship between the experimental results. Regarding the half-life of 1 mg primary threshold-dose antigen as 400 minutes, it can be calculated that the level of antigen decreases to the threshold of the secondary immune response in 46 to 60 hours. When two subliminal antigen doses are given, a 40 hour interval is more effective than a 21 hour interval

Discussion

The development of immunological memory against various antigens and in various species has been demonstrated by several authors. These data concern mainly 7S type antibodies and are in good agreement. As to 19S type antibodies, the data are contradictory. In some cases only 7S type immunological memory develops [5, 57], in others the development of 19S type memory has also been demonstrated [7, 38]. Neutralizing antibodies produced against phosphorylase in mice are 7S in type, similarly to antibodies neutralizing diphtheria toxin and egg white lysozyme [40].

It is generally accepted that the cellular substrate of immunological memory is the memory cell (immunologically activated, Y cell) which develops from an immunologically competent cell (X cell) as a result of the antigenic stimulus [45, 50].

The kinetics of the development of immunological memory can conveniently be studied by applying two subliminal antigenic stimuli. In such experiments the amount of antibodies produced after the secondary antigenic injection is proportional to the degree of immunological memory. In the present work we have examined the earliest development and the further time-course of immunological memory by determining the relationship between antigen dose and primary and secondary immune response and the effect of the interval between the two stimuli on the plasma antibody level. The antigen doses needed for inducing a pure immunological memory and for giving rise to a secondary immune response were similar in order of magnitude (0.8–8 μg). For inducing a primary antibody production, about two exponents higher doses were needed (1 mg). The high difference between the threshold values for primary and secondary immune response was attributed to the rapid, non-specific elimination of the antigen from the circulation. If the estimated half-life of the threshold dose of primary immune response is 400 minutes, the antigen level decreases in 46 to 60 hours to the threshold of secondary immune response. This time may be regarded as the time of X–Y transformation. On the basis of our results (Figs 1–4) Fig. 6 has been prepared which schematically summarizes the assumed association between the time of X–Y transformation, the threshold doses for primary and secondary immune responses, the interval between the two subliminal antigen doses and the non-specific elimination of the antigen.

Although the degree of non-immunological degradation of phosphorylase is not exactly known, it may be assumed that this process proceeds parallel with the disappearance of active phosphorylase from blood. This consideration seems to be confirmed by the dose and time dependence of the primary and secondary immune responses. The ineffectiveness of primary antigen doses lower than 1 mg is probably due to the rapid elimination of the antigen as

after an interval the reinjection of small, subliminal doses results in antibody production. When two subliminal doses had been given, a two-day interval was more effective than a one-day interval, because in the first case antibody production was observed 8 days after the primary dose, while in the second no antibodies were detected after the same period. The fact that in this experiment antibodies were shown on the 7th day only when the interval between the two injections had been 2 or more days may have been due to that priming for secondary antibody response needs at least two days.

This finding is in accordance with the assumption that antibody production is a two-stage process [3, 9, 10, 26, 36, 45, 47—49] which consists essentially of the development of competent cells (X) into memory cells (Y), then into antibody-producing cells (Z). The evidence for this has recently been summarized and discussed by STERZL and SILVERSTEIN [50] and MAKINODAN and ALBRIGHT [28a]. The memory cells need a repeated contact with the antigen to be able to develop into antibody-producing cells [54].

Our data strongly suggest that the early development of Y-cells in mice primed with phosphorylase takes 2 to 2.5 days. This observation confirms the finding of CRUCHAUD and COONS [9] who showed that priming in mice with diphtheria toxoid was inhibited by chloramphenicol only if the antibiotic treatment had been commenced simultaneously with the first antigen injection. Chloramphenicol was ineffective if it was given 48 to 72 hours after the primary antigen dose. It was concluded from this observation that priming is complete in 48 to 72 hours.

More recently VISCHER and STASTNY [59] have studied the early development of immunological memory in rabbits. In ^3H -thymidine incorporation experiments in tissue cultures they showed that the early development of immunological memory took 2 to 4 days.

Another important problem is the further time-course of the immunological memory once it has developed. Observations generally agree that after the primary antigenic stimulus the secondary immune response increases for a while [2, 8, 13, 17, 18, 24, 25, 27, 30, 31, 37, 39, 41, 44, 46].

BARR and LLEWELLYN-JONES [4] performed systematic investigations into the effect on the antibody level of the interval between two doses. They immunized guinea pigs with diphtheria toxoid adsorbed on aluminium phosphate. The secondary immune response increased until the 4th to 6th month after the primary dose then decreased slowly.

FECSIK, BUTLER and COONS [12] inoculated mice with purified diphtheria toxoid containing no adjuvant. They administered the secondary antigen injection 10, 20, 40, 80 and 160 days after the primary one. The secondary immune response increased till the 40th day then remained at a relatively stable high level.

More recently JILEK and STERZL [20] have immunized mice with various

doses of sheep erythrocytes and gave the secondary dose 1, 2, 3, 5, 8, 12 and 19 months after the first injection. The maximum secondary immune response was reached in the 5th month for haemolytic and in the 8th month for haem-agglutinating antibodies.

For examining the development of immunological memory *in vivo*, the ideal antigen would theoretically consist of one component, persist for a short time and exert no prolonged effect. Phosphorylase seems to meet these requirements.

By examining the effect of the interval on plasma antibody levels of two subliminal antigen doses conclusions can be drawn as to the time-course of immunological memory. There are experimental data that the plasma antibody level bears a linear relationship to the number of antibody-producing cells [19, 29, 58]; accordingly, from the rise in plasma antibody level an indirect conclusion can be drawn as to the increase in the number of antibody-producing cells. In cell-transfer experiments it has been shown that spleen cell suspension from optimally primed mice contains 100 times more progenitor cells than the corresponding preparation from nonprimed mice [1]. The assumption that the memory cells begin to multiply after the primary antigenic stimulus [18] seems, accordingly, acceptable [50]. The multiplication of memory cells between the first and second antigen injection was confirmed by NOSSAL and MÄKELÄ [34]. In view of these data the time-course of immunologic memory probably reflects the kinetics of Y-cell multiplication.

An explanation at the cellular level of the above kinetic data is as follows. After the antigenic stimulus the competent cells need 2 to 2.5 days to develop into memory cells (X—Y transformation, priming). The memory cells, if they are not brought into repeated contact with antigen, begin to multiply after a lag phase of 2 to 8 days. The multiplication phase lasts from the 8th to the 20th day. From the 20th day onward the number of memory cells would remain practically constant. This latter would be limited only by the half-life of Y-cells or a dynamic equilibrium between the destruction and formation of Y-cells.

The present data indicate that the increase of potential immunity is not a prolonged steady process but is divided into well-defined stages. In order to distinguish between these stages it seems advisable to use the terms Y lag-, Y exponential- and Y stationary-phases, because these stages are presumably connected with the multiplication of Y cells.

Fairly wide ranges (6 weeks to 8 months) have been described for the interval between two antigenic stimuli the prolongation of which yields no further increase in immune response. Thus it seems advisable to discuss some factors which may influence the result of such experiments and may explain the difference between the results.

1. Persistence of antigen. If the antigen is present in the body for longer periods of time, either because of its special nature or because of its binding to depot-adjuvants, the X—Y transformation and the multiplication of Y clones becomes prolonged.

2. The presence or absence of "adjuvanticity" of the antigen [11] may also influence the kinetics of the development of immunological memory.

3. The use of multiplex (multimolecular) antigens complicates the evaluation of results. The results were different, for example, when antibodies to red blood cells were examined in a haemolytic and in a haemagglutinating system [20].

4. High doses of the antigen at the secondary stimulus. This does not only make the existing memory cells to develop into antibody-producing cells but may also induce a new primary reaction (X—Y—Z transformation).

5. Antibody production after the primary stimulus. The effect of primary antibodies on the development of immunological memory cannot be excluded. Antibodies given passively may exert inhibitory [14, 32, 33, 42, 43, 55, 60] or stimulatory [35, 51, 53, 56] effects. An antigen-antibody complex may be formed as a result of the interaction between the primary antibody and the persistent or secondary antigen. An antigen-antibody complex may stimulate X—Y transformation even when excess antibodies are present. The result of this effect may be the prolongation of the whole process.

6. Changes in the immunoglobulin-type of antibodies in the course of immunization. These may cause difficulties in comparing early and late values if type determinations have not been performed.

7. Species differences between the immunized animals may also be responsible for differences in results.

Our finding that the temporal increase of potential immunity is essentially complete within 3 weeks, as opposed to other data in the literature, might be explained, among others, as follows. (1) A rapid and nonspecific elimination of phosphorylase from blood plasma and, as a result, a presumably short antigen persistence (no prolonged effect); (2) we applied antigen doses that were subliminal in themselves (the influencing effect of primary antibodies was not exerted); (3) finally, the neutralizing antibodies were of a relatively homogeneous 7S population.

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DIFFERENTIATION OF MYCOBACTERIUM TUBERCULOSIS AND MYCOBACTERIUM BOVIS

By

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(Received April 24, 1968)

Summary. A total of 104 strains isolated from patients with pulmonary tuberculosis has been examined. With nitrate, niacin and intracutaneous rabbit tests 101, 95 and 103 cultures, respectively, gave a reaction characteristic of *M. tuberculosis*. As only 21 strains corresponded to the phage picture described as characteristic of *M. tuberculosis*, it has been concluded that animal experiments and biochemical tests are more suitable for differentiation between the two species.

The epidemiological and microbiological importance of the differentiation between *Mycobacterium tuberculosis* and *M. bovis* is shown by the fact that in recent years several new methods such as the niacin test [1], the nitrate reductase reaction [2], and a phage-typing method [3] were described for the identification of the species in question.

The variation in the incidence of *M. bovis* according to geographical areas is generally attributed to local epidemiological conditions; it may, however, be due also to differences in the diagnostic methods used.

The result of the fight against tuberculosis may be influenced by the number and character of infectious sources. Thus the constant observation of the danger from cattle is very important. In view of these considerations a revision of the bacteriological diagnostic methods seems justified. The present work was undertaken to compare biochemical, animal and phage tests.

Materials and methods

Strains. The examined 104 mycobacterial strains were isolated from patients with pulmonary tuberculosis. Each sample was cultured by the common routine method [4] in two tubes of Sula [5] and in two tubes of Löwenstein—Jensen medium. All strains were sensitive to INH, as tested by CANETTI's method [6] as modified by SZABÓ [7]. Two international laboratory strains, H₃₇Rv and Ravenel, and two cultures isolated from cattle with generalized tuberculosis were used for comparison.

Biochemical methods. Nitrate reductase was demonstrated by the semi-quantitative technique of SZABÓ [8] adapted from VIRTANEN's method [2]. To 8 ml 14 day culture grown in modified Sula medium containing 0.1% sodium nitrate 1 ml of Griess—Ilosvay reagent was added. The tubes were read after 10 minutes standing. Nitrate-reducing *M. tuberculosis* cultures were characterized by a pink or red colour; *M. bovis* cultures showed no change in colour.

KONNO's niacin test [1] was performed as modified by MEDVECZKY [9]. To 3 week Löwenstein—Jensen cultures 3% alcoholic benzidine and concentrated cyanogen bromide

solutions were added. Positive reactions characteristic of *M. tuberculosis* were indicated by a purple colour; in *M. bovis* cultures no change was observed.

Animal test. Instead of the classical intravenous test the intracutaneous rabbit reaction found suitable in our institute [10, 11] was used. This method allows the examination of as much as 25 to 30 cultures on one animal. The dorsal skin of the rabbit was depilated, then 0.2 ml aliquots of the suspensions (one loopful culture in 2 ml saline) were injected intracutaneously. While *M. tuberculosis* caused no lasting ulceration at the site of the injection, *M. bovis* gave rise to a spreading ulcer with no healing tendency. Observation of the animals lasted for 8 weeks. The reactions were read at 2 weekly intervals.

Phage typing. In order to standardize mycobacterial phage typing, in 1964 the WHO organized an international team under the guidance of Drs REDMOND and SULA. Work on the improvement of the methods, in which our laboratory takes an active part, is in progress. The present examinations were performed by REDMOND's method [12] using phages DS6A, GS4E, AG1 and GS7 in the appropriate routine test dilutions (RTD). Culturing and typing of the strains were carried out on a special medium designed for this purpose. Undiluted phage suspensions were used as controls. The strains were differentiated according to the patterns shown in Table I.

Table I

Strains	Phages			
	DS6A	GS4E	AG1	GS7
<i>M. tuberculosis</i>	+	+	+	—
<i>M. bovis</i>	+	—	+	—
<i>M. kansasii</i>	—	—	+	—

Results

Table II shows that *M. tuberculosis* was much better characterized by biochemical tests and animal inoculation than by phage-typing. With the nitrate, niacin and intracutaneous rabbit tests 101, 95 and 103 strains, respectively, gave a reaction characteristic of *M. tuberculosis*. With undiluted phage suspensions 45 strains, with RTD phage suspensions 81 strains reacted as *M. bovis*. Data for cultures shown to be *M. bovis* by phage-typing are presented in Table II.

Table II

Distribution of strains according to various examination methods

		Nitrate test	Niacin test	Animal test	Phage-typing	
					RTD	Undiluted
104 strains from routine material	<i>M. tuberculosis</i>	101	95	103	23	59
	<i>M. bovis</i>	2	6	1	81	45
	Doubtful	1	3	—	—	—
Control strains	H ₃₇ Rv	+	+	—	human	human
	Ravenel	—	—	+	bovine	bovine
	Strain 1	—	—	+	bovine	bovine
	Strain 2	—	—	+	bovine	bovine

Note. Strains 1 and 2 were isolated from cattle.

Table III

Identification by other methods of strains shown to be M. bovis by phage-typing

Identified as <i>M. bovis</i> by phage-typing	Number of bovine strains identified as <i>M. tuberculosis</i> by phage-typing			
	Nitrate reduction and niacin test	Nitrate reduction and rabbit test	Niacin test and rabbit test	Nitrate reduction, niacin test and rabbit test
Routine test dilution	73	78	75	73
Undiluted phage	41	43	42	40

Note. Strains giving discrepant results within the above groups have not been included.

It is evident from Table III that typing with RTD and undiluted phage frequently yielded results different from those shown by the other examination methods. With RTD more contradictory data were obtained than with undiluted phage suspension.

One out of the 104 strains reacted in all tests as *M. bovis*. This finding corresponds to data estimating the incidence of *M. bovis* as 1%. With nitrate reductase test 2, with niacin test 6 strains reacted as *M. bovis*.

Discussion

Phage-typing and other tests used for the differentiation of *M. tuberculosis* and *M. bovis* have given discrepant results. MANKIEWICZ and DERNUET [13] found that only 50% of their strains identified as *M. bovis* with REDMOND's phage-typing method belonged to this species according to the intravenous rabbit test. In our examinations 77% of cultures identified as *M. tuberculosis* by the intracutaneous rabbit test behaved as *M. bovis* on the basis of phage-typing. The discrepancy between the biochemical and animal tests and phage-typing was less marked when undiluted phage suspension was used instead of RTD. MANKIEWICZ and DERNUET concluded that *M. tuberculosis* did not behave as a uniform species when tested by the phage method. BATES and FITZHUGH [14] using similar or identical (DS6A and GS4E) test phages were unable to demonstrate any correlation between the niacin test and phage-typing. They divided *M. tuberculosis* on the basis of phage sensitivity into 3 groups.

According to our examinations these methods gave identical results in the overwhelming majority of cases. Contradictory findings in a slight fraction of cases indicate that it is advisable to combine the different methods. In our opinion the type can be determined only if identical results are yielded by several methods. In the case of a small number of contradictory results the type cannot be established with certainty.

In our opinion the phage-typing method will be suitable for a further division of *M. tuberculosis*. The phage-typing method used at present will be suitable for the differentiation of *M. tuberculosis* and *M. bovis* only in possession of new data. For the contradictory findings the inhomogeneous composition of the strains may be responsible. It is hoped from our examinations in this direction that the phage-typing method will be improvable in this manner.

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EFFECT OF RAPHANIN ON THE MULTIPLICATION OF SOME RNA AND DNA VIRUSES IN, AND INTERFERON PRODUCTION BY, CULTURED CELLS

By

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(Received May 2, 1968)

Summary. The effect of raphanin on the multiplication of pseudorabies, vaccinia, Semliki forest, Sindbis, vesicular stomatitis and chikungunya viruses and on the interferon production by chick embryo fibroblasts was examined. Multiplication of DNA viruses was found to be inhibited to a higher degree than that of the RNA viruses. Based on its inhibitory effect on interferon production it is concluded that the antiviral effect of raphanin is due to its effect injuring cell metabolism.

In 1947 IVÁNOVICS and HORVÁTH [1] prepared an antibiotic substance from radish (*Raphanus sativus*) seeds and termed the substance raphanin. Raphanin intensely inhibited the multiplication of a number of pathogenic bacteria, but owing to its high toxicity could not be used for therapeutic purposes. One year later SCHMIDT and KARRER [2] prepared an antibiotic substance, called by them sulphoraphen, from radish seeds. KOCZKA and IVÁNOVICS [3] identified sulphoraphen with raphanin.

Considering that several substances originating in higher plants have proved to inhibit viral reproduction [4, 5], we initiated experiments to demonstrate if nontoxic concentrations of raphanin exerted an antiviral effect in chick embryo fibroblast (CEF) cultures.

Materials and methods

Cell cultures. CEF monolayers were prepared from 11-day-old chick embryos in tubes and in Petri dishes 60 mm in diameter. The cells obtained by trypsin digestion were suspended in a Gey solution to which 5% calf serum, 0.4% lactalbumin hydrolysate and 8% 0.1 M Tris-HCl buffer pH 8.1 had been added. In each tube 1.5×10^6 cells in 1 ml, in each Petri dish 5 ml cell suspension containing 5×10^6 cells per ml were sedimented. The cell cultures were incubated at 37°C for 24 hours before use.

Viruses. The vaccinia virus strain Budapest of the Phylaxia State Serum Institute, Budapest, was maintained in serial passages in HeLa cells. The pseudorabies virus strain originated from Dr. I. BÉLÁDI (Szeged), the Sindbis virus from Dr. ION GRESSER (Boston), the Semliki forest virus from Dr. J. S. PORTERFIELD (London). These viruses were propagated in CEF cells. The vesicular stomatitis virus obtained from Dr. M. GREŠIKOVÁ (Bratislava) and the chikungunya virus obtained from Dr. A. ISAACS (London) was maintained in HeLa cells and in mouse brain, respectively.

Preparation of raphanin. The method described by IVÁNOVICS and HORVÁTH [1] was applied. The stock solution of raphanin contained 10 mg/ml, its pH was adjusted to 5.0 with 0.01 M HCl. The stock solution was diluted in the nutrient medium.

Microbiological assay of raphanin. To determine the antibacterial activity of raphanin preparations, 10^6 cells/ml *E. coli* B were incubated in the minimal medium at 37°C. Twenty-five μg of the preparation per ml medium completely prevented bacterial growth.

Toxicity assay. Graded doses of raphanin were added to CEF suspension obtained by trypsin digestion. The suspensions distributed in glass tubes were placed in a thermostat of 37°C and controlled under the microscope every day for 4 days. The following phenomena were taken into account: attachment of cells to the glass; growth of cells; rate of multiplication; life-span of the cells. Any change in these phenomena as compared to the control, was considered a toxic effect.

A reduced interferon-productive capacity, suggestive of an injured cell metabolism, was also regarded as an indicator of the toxic effect [6].

Testing of virucidal effect. One mg raphanin was added to 1 ml virus suspension containing 2×10^6 PFU/ml. Virus suspensions thus treated and control ones were incubated in a water bath of 37°C for 3 hours and subsequently titrated by the plaque method.

Testing for antiviral effect. Three methods were applied.

(1) Raphanin was added to tube cultures at a subtoxic level as indicated by cytomorphological test. At the same time virus was added at a rate of 0.01 PFU per cell. The cultures were incubated at 37°C for 48 hours and examined microscopically every day. The virus in the culture media was determined after 48 hours (Sindbis virus) or 72 hours (pseudorabies virus) by plaque titration.

(2) Monolayer cultures were infected with 10^3 PFU of virus each. After an adsorption period of 3 hours at 37°C the cell layer was washed with 2×5 ml Gey solution and covered with a Gey solution enriched with 0.25% lactalbumin hydrolysate, 5% calf serum, 15% 0.1 M Tris-HCl buffer of pH 8.1, 0.008% neutral red and 1.35% agar. When the overlay had solidified, wells 4 mm in diameter were bored and 0.05 ml raphanin solution of variable concentration was dripped in each. The cultures were incubated at 37°C and examined whether plaque-free zones had developed around the holes [7].

(3) On cell monolayers 10^2 PFU of virus was allowed to adsorb for 3 hours. Subsequently a subtoxic quantity of raphanin was added to the overlay medium and the cultures were covered. When plaque had appeared, the percentual plaque reduction was calculated [4].

Testing of the raphanin-inactivating effect of cysteine. One hundred PFU of pseudorabies virus was added to each of cell monolayers, allowed to adsorb for 3 hours at 37°C and covered with an overlay containing 30 $\mu\text{g}/\text{ml}$ raphanin and various concentrations of cysteine (Reanal, Budapest). The degree of raphanin inactivation was estimated from the change in the plaque-inhibiting effect as compared with the inhibition in the cysteine-free control cultures.

Interferon assay was carried out as described earlier [6].

The effect of raphanin on interferon synthesis was determined by adding to tube cultures various dilutions of raphanin together with the virus inoculum. Interferon production was assayed by using Sindbis virus [6].

Results

Toxicity of raphanin. In tube cultures 70 to 80 $\mu\text{g}/\text{ml}$ in the medium considerably injured the cells, whereas 40–50 $\mu\text{g}/\text{ml}$ did not change their cytomorphology. The latter concentration did not reduce the life-span of the cultured cells even under the agar overlay. A concentration of 70 to 80 $\mu\text{g}/\text{ml}$ was toxic in this system. In the agar-diffusion test a raphanin concentration of 1500 $\mu\text{g}/\text{ml}$ had to be dripped into the well to induce a toxic effect.

Virucidal effect of raphanin. Raphanin at the concentrations tested proved to be non-virucidal.

Antiviral effect of raphanin. In tube cultures 35 $\mu\text{g}/\text{ml}$ raphanin definitely inhibited the multiplication of pseudorabies virus, whereas Sindbis virus was hardly inhibited at that concentration (Table I).

Table I
*Effect of raphanin on the multiplication
of pseudorabies and Sindbis viruses*

Raphanin $\mu\text{g/ml}$	Pseudorabies virus		Sindbis virus	
	CP effect*	log. PFU/ml	CP effect**	log. PFU/ml
0	++++	8.08	++++	8.75
10	++++	8.12	++++	8.53
20	++	6.81	++++	8.60
35	±	5.70	++++	8.19

* 72 hours after inoculation

** 48 hours after inoculation

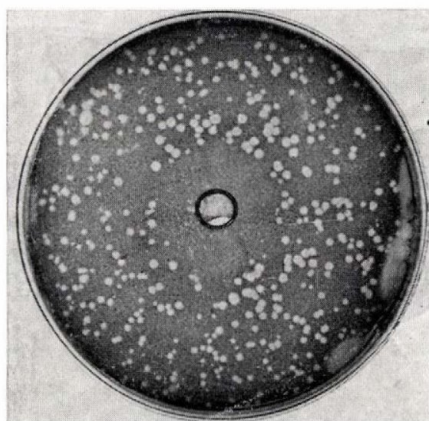


Fig. 1. Effect of 1000 $\mu\text{g/ml}$ raphanin on plaque formation by pseudorabies virus (agar-diffusion-test)

The agar-diffusion test yielded similar results. Fig. 1 shows the plaque reduction by a solution containing 1000 $\mu\text{g/ml}$ raphanin, using pseudorabies virus as the test organism. Plaque formation by Sindbis virus was not reduced even at higher concentrations.

The results of the plaque-reduction tests have shown that among the RNA viruses tested only the Semliki forest virus was considerably affected by raphanin. Plaque-formation by the other RNA viruses was not or only slightly reduced (Table II). The results obtained with the DNA viruses vaccinia and pseudorabies are shown in Table III. These viruses were inhibited by raphanin to a greater extent than the Semliki forest virus.

If raphanin-treated monolayers were incubated after inoculation longer than indicated under Materials and methods, namely for 96 hours, new minute plaques became visible. This phenomenon was most pronounced in the case

of pseudorabies virus, while vaccinia virus did not form such late plaques (Tables II and III).

Table II

Effect of raphanin on plaque formation by four RNA viruses

Raphanin $\mu\text{g/ml}$	Plaque-count reduction in per cent							
	48	96	48	96	48	96	72	96
	hours after inoculation with							
	Sindbis virus		Semliki forest virus		Vesicular stomatitis virus		Chikungunya virus	
0	0	0	0	0	0	0	0	0
15	0	0	3	0	0	0	0	0
25	8	3	46	15	0	0	2	0
35	31	7	89	72	4	0	27	10

Table III

Effect of raphanin on plaque formation by pseudorabies and vaccinia virus

Raphanin $\mu\text{g/ml}$	Plaque-count reduction in per cent			
	72	96	72	96
	hours after inoculation with			
	pseudorabies virus		vaccinia virus	
0	0	0	0	0
10	0	0	2	2
15	20	5	8	8
25	100	53	93	93
35	100	96	100	100

Effect of cysteine on raphanin. Raphanin is rapidly inactivated by various thiol compounds [8]. Therefore, the effect of cysteine was examined. Fig. 2 shows the inactivating effect of cysteine at different concentrations on 30 $\mu\text{g/ml}$ raphanin.

Inhibition of interferon synthesis. Interferon synthesis by CEF cells was intensely inhibited by raphanin (Table IV). A concentration of 10 $\mu\text{g/ml}$ was inhibitory and by 35 $\mu\text{g/ml}$ synthesis was practically blocked.

Table IV

Effect of raphanin on interferon synthesis induced by Sindbis virus in CEF cell

Raphanin $\mu\text{g/ml}$	Interferon titre
0	64
5	64
10	32
15	32
25	8
35	2
50	0

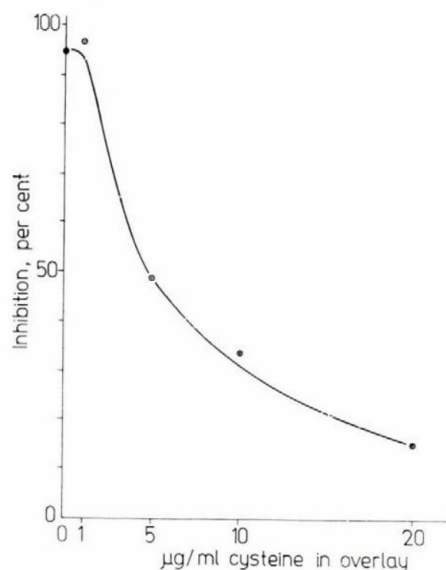


Fig. 2. Inactivating effect of cysteine on 30 $\mu\text{g/ml}$ raphanin.
(Experiments with pseudorabies virus)

Discussion

According to the present results the reproduction of pseudorabies and vaccinia virus was definitely, that of Semliki forest virus to a lesser extent inhibited by raphanin. Sindbis, vesicular stomatitis and chikungunya viruses were not or scarcely influenced by raphanin at the concentrations applied.

The appearance of new plaques during prolonged incubation suggested that the effect of the raphanin concentrations applied in the present experiments was not completely irreversible.

The inhibition of interferon production by raphanin at low concentrations indicated that some metabolic processes of the host cell are intensely injured even by such low concentrations. According to MOLNÁR [9] the effect of raphanin is based on the inhibition of thiol enzymes. Inhibition of the multiplication of some viruses appears to be attributable to this phenomenon. On the basis of the present results it may be assumed that the reproduction of DNA viruses is more sensitive to the inhibition of SH-containing enzymes than the reproduction of RNA viruses.

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THE EFFECT OF SORBIC ACID ON THE GROWTH OF THE YEAST *PROCANDIDA* (*CANDIDA*) *ALBICANS*

By

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(Received May 6, 1968)

Summary. The inhibitory effect of potassium sorbate on the growth of *Procandida albicans* in different incubation systems has been examined. Determination of the growth rate constant, lag phase and final population density has shown that independently of the composition of the medium the inhibitory effect of sorbate is stronger under anaerobic than under aerobic conditions. The decreased inhibition at aerobic incubation was attributed to a detoxication of sorbate by the yeast culture. The degree of this aerobic detoxication varied with the composition of the medium. Cysteine and glutathione exerted an effect quite opposite to that expected on the basis of the literature; instead of antagonizing the growth inhibitory effect of potassium sorbate, they increased the degree and duration of inhibition by decreasing the detoxication of the inhibitory agent.

Sorbic acid (2,4-hexadienic acid) is a fungistatic substance particularly effective against yeasts and moulds. It is widely used in food industry [4, 7—9] and there are several data concerning its concentration inhibiting the growth of yeasts [1, 2, 11, 13]. However, no systematic examinations have been carried out as to the kinetics of its growth inhibitory effect. In the present paper some studies into this problem are described.

Materials and methods

Sorbic acid was used as potassium sorbate (Farbwerke Hoechst A. G.). Determination of potassium sorbate concentration in the cultures and control measurement of the added substance were performed as described by LUCKMANN and MELNICK [6] and MELNICK and LUCKMANN [10].

Pc. albicans strain 85/1957 was isolated in our laboratory and maintained on CSILLAG's molasses agar slants [3]. Cultures obtained on the same medium after 48 hours incubation were suspended in saline, set to a density corresponding to $5-8 \times 10^6$ cells per ml and used as inocula. Growth was examined in 4 different media: 1. Sabouraud glucose broth with organic nitrogen source (Witte peptone) adjusted to pH 5.6 and supplied with a yeast extract as a vitamin source (1000 g baker's yeast cake mixed with 1200 ml water, autoclaved at 121 °C for 30 minutes; 0.025 ml of this was added to 10 ml medium before inoculation); 2. Medium 1 without yeast extract; 3. Synthetic medium with inorganic nitrogen source: glucose, 40 g; $(\text{NH}_4)_2\text{SO}_4$, 5 g; KH_2PO_4 , 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; distilled water to 1000 ml; pH 5.6; yeast extract as described above; 4. Medium 3 without yeast extract.

Aerobic and anaerobic cultivation was performed under shaking and under static conditions, respectively. Ten ml amounts of the media were distributed into T-shaped tubes and aerated on a home-made tilting shaker set at 60 r.p.m. and a vertical amplitude of 5 cm at the end of tubes. As before each photometric measurement the test tubes were shaken, until a constant optical density had been reached, the anaerobic cultures were in reality only semi-anaerobic ones. Both aerobic and semi-anaerobic incubations were carried out at $22 \pm 2^\circ\text{C}$.

The degree of growth was determined turbidimetrically in a model FEK-M photo-electric colorimeter by placing the test tubes in cuvettes filled with distilled water as described by VAS [15]. Optical density was expressed as population density by the use of a calibration curve prepared by direct counting in a Bürker chamber. By plotting the logarithm of population density against time, the growth rate constant (k) was calculated from the slope of the straight part of the curve. The effect of different experimental conditions was always measured by comparing the growth rate constant of the individual tubes to that of the control culture and was expressed by the ratio of the value of the respective culture and of the control (relative k). In examining the kinetics of growth, on the basis of HINSELWOOD's suggestion [5] the changes of the duration in the lag phase and in the final population density were also taken into consideration. The importance of these factors has been emphasized also by VAS [14]. The duration of lag phase was estimated by extrapolating graphically the straight part of the growth curve, drawing a line at population density $8 \times 10^6/\text{ml}$ and reading the interval between zero time and the time indicated by the intersection of the two lines. The final population density was calculated from photometric readings performed for aerobic conditions after 72 hours and for semi-anaerobic ones after 96 hours.

Results

The effect of potassium sorbate on the growth of *Pc. albicans* was examined in 8 experiments representing different combinations of the following three factors: 1. mode of incubation (aerobic shaken or semi-anaerobic

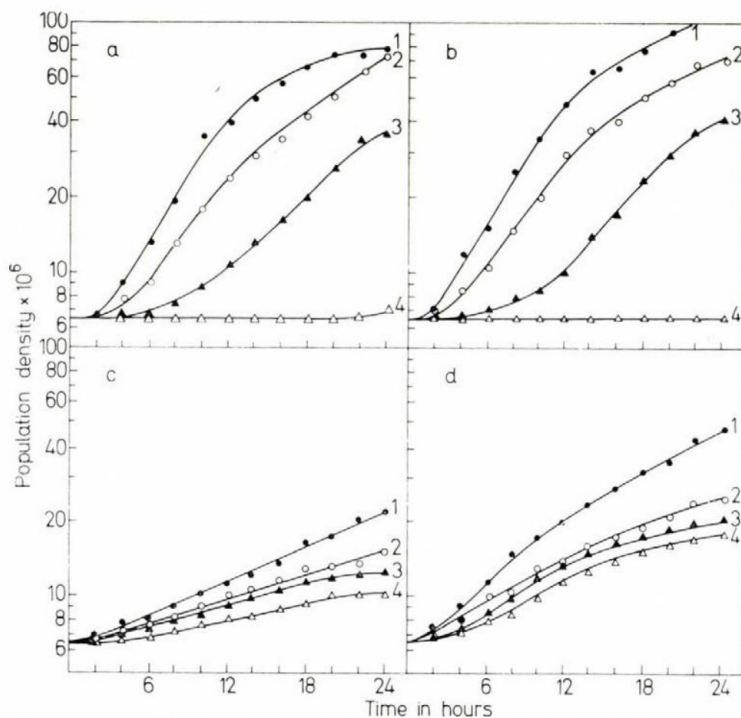


Fig. 1. Effect of potassium sorbate at various concentrations on the aerobic growth of *Pc. albicans*. a — Sabouraud glucose medium; b — Sabouraud glucose medium supplemented with yeast extract; c — synthetic medium; d — synthetic medium supplemented with yeast extract. 1 — control; 2 — 250 μg sorbate/ml; 3 — 500 μg sorbate/ml; 4 — 1000 μg sorbate/ml

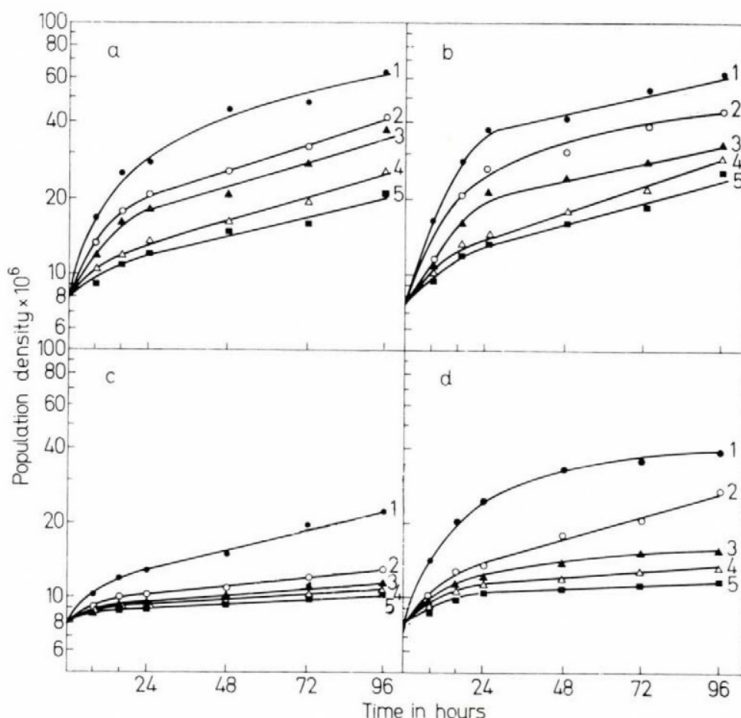


Fig. 2. Effect of potassium sorbate at various concentrations on the anaerobic (semi-anaerobic) growth of *Pc. albicans*. a, b, c and d: see Fig. 1. 1 — control; 2 — 100 μg sorbate/ml; 3 — 150 μg sorbate/ml; 4 — 200 μg sorbate/ml; 5 — 250 μg sorbate/ml

static cultures); 2. growth in the presence of organic or inorganic nitrogen source (Sabouraud glucose broth and synthetic medium); 3. growth in the presence and absence of vitamins (yeast extract). On the basis of preliminary experiments the sorbate concentrations applied were 250, 500 and 1000 $\mu\text{g}/\text{ml}$ for shaken and 100, 150, 200 and 250 $\mu\text{g}/\text{ml}$ for static cultures. The results of growth experiments in these systems are shown in Figs. 1 and 2.

Although the characteristics of the growth of *Pc. albicans* seem to vary with the incubation system, the effect of sorbate could be properly evaluated by taking into account the relative values of the parameters.

Changes in the relative k values in aerobic cultures as a function of different sorbate concentrations are presented in Fig. 3. The dose-response curve was definitely steeper for cultures growing in the presence of organic nitrogen, and the effect of sorbate was not appreciably influenced by the presence or absence of vitamins. When the cultures were incubated under anaerobic conditions (Fig. 4), neither the nitrogen nor the vitamin source exerted a significant effect on the results.

Potassium sorbate was found to prolong the lag phase in aerobic cultures (Fig. 5). The inhibitor, especially at the highest concentration used, retarded

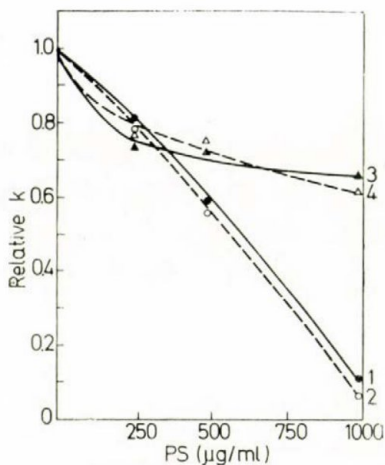


Fig. 3. Effect of potassium sorbate concentration on growth rate of *Pc. albicans* in aerobic culture. 1 — Sabouraud glucose broth; 2 — Sabouraud glucose broth supplemented with yeast extract; 3 — synthetic medium; 4 — synthetic medium supplemented with yeast extract

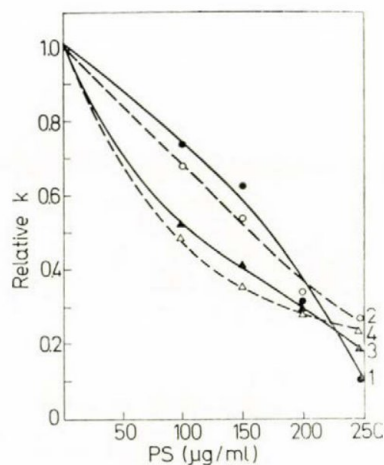


Fig. 4. Effect of potassium sorbate concentration on growth rate of *Pc. albicans* in anaerobic (semi-anaerobic) cultures. The curves are as in Fig. 3

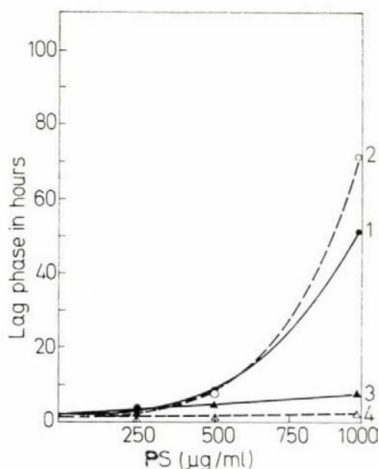


Fig. 5. Effect of potassium sorbate concentration on the lag phase in aerobic culture. The curves are as in Fig. 3

the start of growth in the organic nitrogen medium more definitely than in the synthetic medium. The effect of sorbate on the lag phase in anaerobic cultures could not be evaluated because, in order to avoid a frequent shaking of the cultures, results were read at 8 and later at 12-hour intervals.

As to final population density, sorbate at 1000 $\mu\text{g/ml}$ concentration exerted a drastic inhibitory effect in Sabouraud glucose broth (Fig. 6). In synthetic medium the dose-response curve was linear and there was a definite decrease in the inhibitory effect when the medium had been supplemented with vitamins. As most of the anaerobic cultures showed a more or less definite growth after 96 hours, the differences in final population density could not be evaluated. However, the growth curves (Fig. 2) clearly indicated that in synthetic media in the presence of sorbate at concentrations of 150 $\mu\text{g/ml}$ or more the population density did not change considerably after 24 hours, and was inversely proportional to the sorbate concentration.

After longer incubation periods, although after prolonged lag phases, a definite growth started even at higher sorbate concentrations. Sorbate concentration was therefore checked not only at the start but also at the end of the experiments. In anaerobic cultures there was no change in the amount of sorbate; in aerobic cultures, however, as it could be expected from the changes in the growth curves, the initial sorbate concentration decreased considerably (Table I).

Table I

*Decrease of potassium sorbate concentration
in aerobic cultures of Pc. albicans*
Initial potassium sorbate concentration 1000 $\mu\text{g/ml}$

Medium	Decrease of potassium sorbate concentration, per cent	
	72 hours	96 hours
Synthetic	43	74
Synthetic + vitamin source	80	100
Sabouraud	—	30
Sabouraud + vitamin source	—	15

Since potassium sorbate inhibits SH-enzymes in vitro and this inhibition can be antagonized with cysteine and other thiols [16, 17], it has been supposed that the same was true for the effect of the agent on growth in vivo. The experiments were performed with a moderately inhibiting sorbate concentration (500 $\mu\text{g/ml}$) and equimolar (3.33 mM) cysteine or reduced glutathione (GSH) concentration; GSH was applied also at 10 and 100 times lower concentrations (Fig. 7). In contrast to the expected result, GSH at 0.33 mM concentration or over increased the inhibitory effect of sorbate considerably or even up to the total inhibition. Cysteine at 3.33 mM concentration exerted an effect identical with the corresponding GSH level. In accordance with the

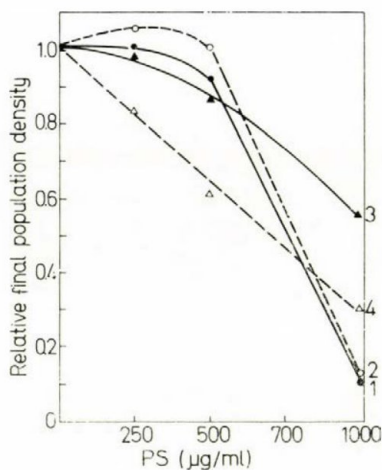


Fig. 6. Effect of potassium sorbate on the final population density in aerobic culture. The curves are as in Fig. 3

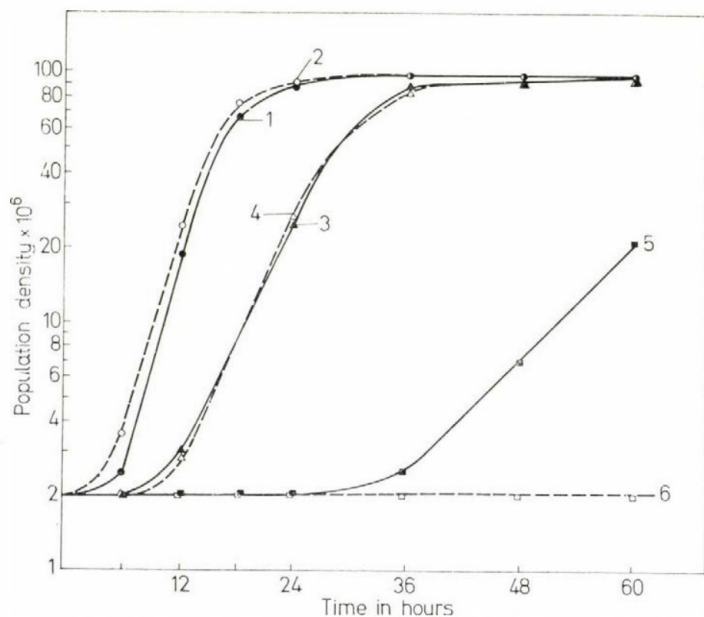


Fig. 7. Synergic effect of potassium sorbate and reduced glutathione (GSH) on the growth of *Pc. albicans*. Aerobic cultures in Sabouraud glucose broth. 1 — control; 2 — GSH controls without sorbate (0.033, 0.33 and 3.33 mM GSH); 3 — 3.33 mM (500 µg/ml) sorbate; 4 — 3.33 mM sorbate + 0.033 mM GSH; 5 — 3.33 mM sorbate + 0.33 mM GSH; 6 — 3.33 mM sorbate + 3.33 mM GSH

increased growth inhibitory effect in the presence of these thiols, the more the cultures were inhibited, the higher was their sorbate content, even after 48 to 60 hours of incubation (Table II).

Table II

*Effect of reduced glutathione (GSH) and cysteine on the decrease of potassium sorbate concentration in aerobic cultures of *Pc. albicans**

Sabouraud glucose broth cultures; initial potassium sorbate concentration, 500 $\mu\text{g/ml}$ (3.33 mM)

Thiol	Decrease of potassium sorbate concentration, per cent	
	48 hours	60 hours
Nil	100	100
0.033 mM GSH	100	100
0.33 mM GSH	42	57
3.33 mM GSH	—	—
3.33 mM cysteine	—	—

Discussion

It may be concluded that independently of the vitamin content and nitrogen source of the medium, the anaerobic growth of *Pc. albicans* was definitely inhibited at small concentrations of sorbate. In anaerobic cultures 250 $\mu\text{g/ml}$ concentration decreased the growth rate by about 80%; in aerobic cultures the decrease at the same concentration was only 20%. At higher sorbate concentrations the degree of inhibition differed according to the nitrogen source. In peptone (Sabouraud) medium 1000 $\mu\text{g/ml}$ sorbate caused about 90%, in ammonium sulphate (synthetic) medium about 35% inhibition. The degree of inhibition was not influenced by the presence or absence of yeast extract applied as a vitamin source. In peptone medium the lag phase was considerably prolonged with the increase of the sorbate concentration. The final population density in the presence of the inhibitory agent at 1000 $\mu\text{g/ml}$ concentration was the lowest in peptone medium. Supplementing of the medium with vitamins influenced the final population density only in synthetic medium.

These results should be interpreted in the light of the changes in sorbate concentration. Sorbate determinations during incubation indicated that under aerobic conditions the concentration of the inhibitory agent gradually decreased. In peptone medium the decrease was considerably less than in synthetic medium and thus the effective sorbate concentration was higher in the former medium throughout cultivation. Thus at a given sorbate concentration the changes observed in the growth of the micro-organism are associated with the degree of the decrease in sorbate concentration which is influenced by the composition and aeration of the medium. Before discussing the effect

of glutathione in detail, it should be noted that the stronger and more durable inhibition shown in peptone medium may have been due to the presence in the peptone of cysteine, glutathione or other substances exhibiting a similar effect. It should be emphasized that sorbate concentration was found to decrease only in aerated tubes. This finding explains the fact that in anaerobic cultures even lower sorbate concentrations exerted a lasting inhibitory effect and, on the other hand, as the concentrations of sorbate remained practically unaltered, there was no difference between the various media as to the inhibitory effect of the agent. Accordingly, in aerobic cultures the composition of the medium is not directly responsible for differences in the inhibition, because it does not influence directly the effect of sorbate but affects only the rate of the decrease in sorbate concentration.

It may be assumed that the decrease in the concentration, or sometimes even the total disappearance of sorbate is associated with the aerobic life activities of the yeast. This process may consist in a "detoxication" of such a degree which already caused an alteration in the sorbate molecule involving the loss of its inhibitory effect and adsorption maximum at 262 m μ . Elucidation of this problem requires further experiments.

According to the data based on experiments *in vitro* it may be supposed that the effect of sorbate *in vivo* is associated with its inhibitory action on SH-enzymes. It seemed therefore justified to expect that in analogy to the elimination of enzyme inhibition *in vitro*, the growth inhibitory effect of the agent can also be antagonized with GSH or cysteine. In contrast, these SH-compounds were found to increase and prolong the inhibitory effect of sorbate. Determinations of the inhibitor in the cultures indicated that this effect of thiols was an indirect one, as it involved only a delaying or hindering of the process of sorbate detoxication. The observations indicate partly that the effect of sorbate *in vivo* differs in mechanism from its effect on enzymes *in vitro*, and partly that the presence of GSH in the system is unfavourable for the microbial detoxication of sorbate. The present data are insufficient for a more detailed discussion of the problem; it should, however, be added that our observations are supported by REHM [12], who found a synergism with cysteine in the inhibitory effect of sorbic acid.

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STRESS REACTION OF MICE TREATED WITH ANTILYMPHOCYTE SERUM*

By

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Summary. Stress-induced lymphopenic reaction decreased or failed to occur in mice treated previously with antilymphocyte serum. The degree of lymphogenic reaction was associated with the absolute lymphocyte count. Animals with low lymphocyte counts showed an increased sensitivity to cold.

Previous studies have indicated that the wasting syndrome induced in mice by neonatal thymectomy was characterized not only by immunological but also by adaptational disturbances. The reaction of thymectomized mice to cold stress was different than that of normal animals: they showed an increased sensitivity to cold and a decreased lymphopenic reaction after the stress. The degree of cold sensitivity and lymphopenic reaction was associated with changes in the absolute lymphocyte count [1].

Administration of antilymphocyte serum (ALS) causes a profound fall in the absolute number of circulating lymphocytes [2]. In the present study the response to various stressors of mice treated with ALS was examined.

Materials and methods

Animals. Inbred 2 to 3-month-old C3H mice weighing 23 to 25 g were used.

Preparation of ALS. Rabbits were immunized with cell suspension prepared from the inguinal and axillary lymph nodes of C3H mice. Preparation of the cell suspension, immunization and absorption of the serum with mouse erythrocytes were performed as described by GRAY *et al.* [2]. Each dose consisted of a freshly prepared suspension containing $80-100 \times 10^6$ cells. For the first dose the cell suspension was diluted with an equal volume of complete Freund's adjuvant (received from the National Institute of Public Health, Budapest). The immune serum was stored at -20°C ; before use it was inactivated at 56°C for 30 minutes then absorbed with C3H mouse erythrocytes.

ALS treatment. The ALS was diluted with an equal volume of saline and one 0.5 ml dose was injected intraperitoneally to each mice. Control mice were treated in a similar manner with normal rabbit serum.

Cold stress. Groups of mice were exposed to 4°C temperature for 4 hours on the day of ALS treatment as well as 1 and 7 days after the injection.

Formalin stress. One day after ALS treatment 0.1 ml of 4% formalin solution was administered subcutaneously.

Determination of absolute lymphocyte count was performed under standardized conditions in blood samples taken from the caudal vein.

The degree of lymphopenic reaction was estimated from absolute lymphocyte counts determined immediately before and 6 hours after the stress.

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Results

The degree and time-course of lymphopenia were examined in 20 treated and 20 control mice. Results are shown in Fig. 1.

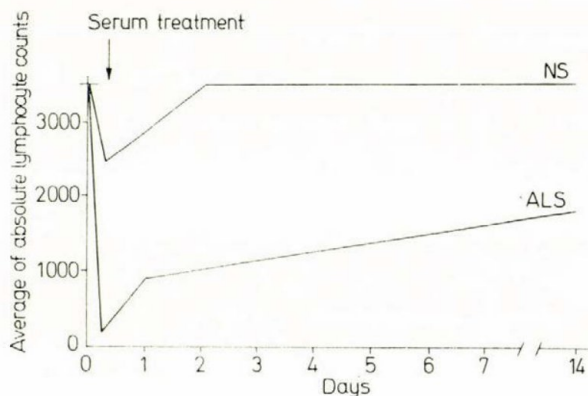


Fig. 1. Average of absolute lymphocyte counts in mice after ALS treatment. NS = normal serum; ALS = antilymphocyte serum

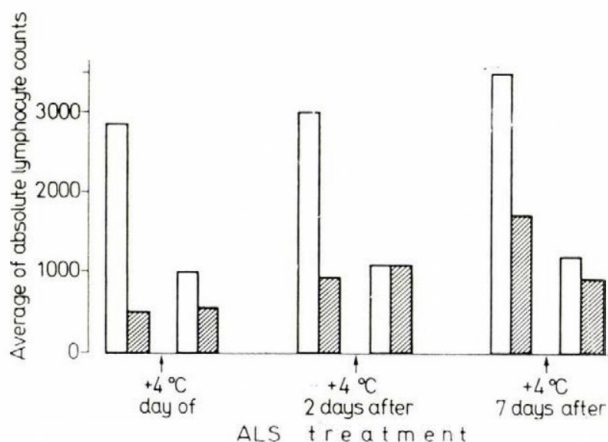


Fig. 2. Absolute lymphocyte count in test and control groups of mice exposed to cold at various intervals after ALS treatment. Clear columns: control animals treated with normal serum; shaded columns: animals treated with antilymphocyte serum

Under the effect of ALS treatment the average absolute lymphocyte count decreased from 3000 to 200–400 in 4 hours after injection; on the next day it increased to 900; by the 14th day the values were still not higher than 50% of the control.

In subsequent experiments the response to stress of ALS-treated mice was examined. Mice were exposed to cold in three groups: 50 mice on the day of ALS treatment, 25 mice each one and seven days after injection. The

number of control animals and the procedures were the same as in the test groups. Changes in absolute lymphocyte counts are shown in Fig. 2.

All control animals showed the lymphopenic reaction described by HARLOW and SELYE [3]; the absolute lymphocyte count decreased from 3000 to 1000 on the average. In mice treated with ALS the results were different. On the day of ALS administration no further decrease was observed in the

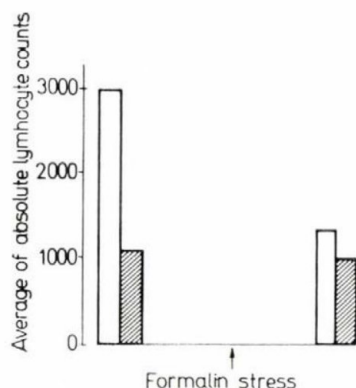


Fig. 3. Average of absolute lymphocyte counts in test and control groups of mice after formalin stress. Clear columns: control animals treated with normal serum; shaded columns: test animals treated with antilymphocyte serum

low absolute lymphocyte count; at the 4th hour of the stress 23 of the 50 treated mice died. In the group exposed to cold one day after ALS treatment no lymphopenic reaction was observed and all animals remained alive. In the group exposed to cold on the 7th day after ALS treatment the lymphopenic reaction was less definite than in the control animals.

The effect of formalin injection given one day after ALS treatment was examined in 20 tests and the same number of control animals. The absolute lymphocyte counts are shown in Fig. 3.

In control animals treated with normal rabbit serum the lymphopenic reaction was marked. The absolute lymphocyte count in ALS-treated mice showed no change; it was about 1000 before and after the stress.

Discussion

The results allowed the following conclusions.

1. The absolute lymphocyte count in mice subjected to stress was about 1000 both in the test and in the control group.
2. In the period when the absolute lymphocyte count was below 1000, the cold stress caused the death of a large number of animals.

3. Lymphopenic reaction developed in ALS-treated mice only when the absolute lymphocyte count had increased over 1000.

These results corresponded to the findings in wasting syndrome developing after neonatal thymectomy [1]. Mice made lymphopenic either by ALS treatment or by thymectomy reacted differently from the control animals. After exposure to cold the lymphopenic reaction failed to develop or was less definite and sensitivity to cold increased markedly. Animals showing the lowest absolute lymphocyte counts (mice exhibiting the severest symptoms of wasting syndrome and mice exposed to cold on the day of ALS treatment) were the most sensitive to cold. Formalin treatment gave similar results: the lymphopenic reaction failed to develop if the absolute lymphocyte count was not higher than 1000.

Our results seem to indicate that, in addition to the adaptive immune response, some other adaptational functions too are associated with the thymus and lymphocyte system. It may be assumed that disorders in the function of the thymus and lymphocyte system are responsible not only for immunological but also for other adaptational disturbances.

It is known that ALS treatment increases the blood corticosteroid level [2]. In subsequent experiments an attempt will be made to elucidate the association between ALS-induced lymphopenia, changes in corticosteroid level, and increased sensitivity to cold.

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THE EFFECT OF GOLD TREATMENT ON LOCAL SHWARTZMAN PHENOMENON

By

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(Received June 21, 1968)

Summary. Aurothioglucose in oil (Solganal B oleosum) in one dose or in two subsequent doses was injected intracutaneously at sites prepared with endotoxin in rabbits. The treatment inhibited or markedly decreased the Schwartzman phenomenon. It has been assumed that locally administered gold salts act on lysosomes or lysosomal enzymes.

THOMAS [1] injected rabbits intracutaneously with rabbit leucocyte homogenate (lysosomes) then intravenously with endotoxin; the result was a haemorrhagic necrosis characteristic of Schwartzman phenomenon. HALPERN [2] confirmed this finding and showed that the necrosis could be prevented with the protease inhibitor Trasylol®. These findings indicate that lysosomes or enzymes released from them play an important role in the development of Schwartzman phenomenon.

PERSELLIN and ZIFF [3] showed in vitro that gold salts accumulated in the lysosome fraction and inhibited the activity of lysosomal enzymes. CHATEL, RICHTER and BOZÓKY [4] observed that prolonged treatment of man with gold salts inhibited the activity of lysosomal enzymes. LÉVAI *et al.* [5] have recently confirmed that gold salts accumulate in the lysosome.

These observations have made us to study the effect of gold on local Schwartzman phenomenon.

Materials and methods

Local Schwartzman phenomenon was produced in 16 rabbits with *E. coli* O111 endotoxin. Bilateral intracutaneous preparatory injections of 0.2 ml endotoxin diluted 1 : 1, 1 : 2 and 1 : 3 were given into the shaven dorsal skin. The reaction was elicited 24 hours later with an intravenous injection of 1 ml endotoxin diluted 1 : 3. Four hours after the preparatory injection each rabbit received 0.1 ml of gold salt into the prepared areas on the right side. Eight rabbits were subsequently given 0.1 ml doses of gold salt at the same site 1 hour before eliciting the Schwartzman reaction.

Aurothioglucose (20%) in oil (Solganal B oleosum — Schering) was used. The controls were injected with 0.1 ml of the solvent oil into the prepared area on the left side.

Results were read 24 hours after administration of the eliciting dose. The skin at the site of the reaction was cut out, fixed in Susa or in 8% formalin and embedded in paraffin. The sections were stained with haematoxylin-eosin.

Results

In 6 rabbits the gold salt was injected into the edge of the oedematous and hyperaemic area around the site of the preparatory injection. Fig. 1 shows that 24 hours after the eliciting injection a semilunar haemorrhagic necrotic reaction developed. This unusual finding was explained by the fact that characteristic Shwartzman phenomenon developed only in that part of the reaction area which had not been infiltrated with the gold salt. When the gold salt had been injected into the centre of the prepared area, a considerably

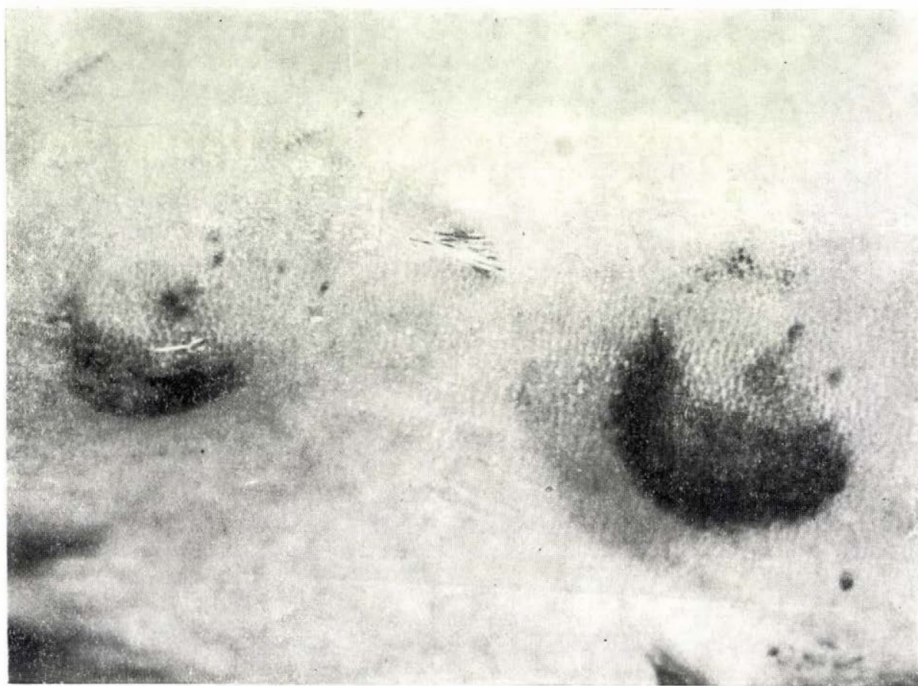


Fig. 1. Local Shwartzman phenomenon 24 hours after the eliciting injection. Effect of gold salt injected into the edge of the prepared area

decreased Shwartzman phenomenon was observed. Two subsequent doses of gold salt completely prevented the Shwartzman reaction (Fig. 2). The solvent had no effect whatever.

Histological sections prepared from areas treated with the solvent oil were characterized by thrombus-like capillary obliteration, necrosis and round cell infiltration (Fig. 3). In contrast, in gold salt-treated areas thrombosis was absent; the round cell and leucocytic infiltration can be interpreted as a response to the preparatory endotoxin injection (Figs 4 and 5).

The findings indicate that locally administered gold salt in one dose markedly decreases, in two subsequent doses completely inhibits the development of local Schwartzman phenomenon.



Fig. 2. Local Schwartzman phenomenon 24 hours after the eliciting injection. *A* = two subsequent injections of oil; *B* = two subsequent injections of gold salt

Discussion

Recent investigations performed in our Institute have confirmed the basic role of lysosomes in the Schwartzman phenomenon. It has been shown that hyperbaric oxygenation increases the severity and rate of development of haemorrhagic necrosis [6]. The effect is probably due to the fact that hyperoxic conditions increase the harmful effect of endotoxin on the lysosomal

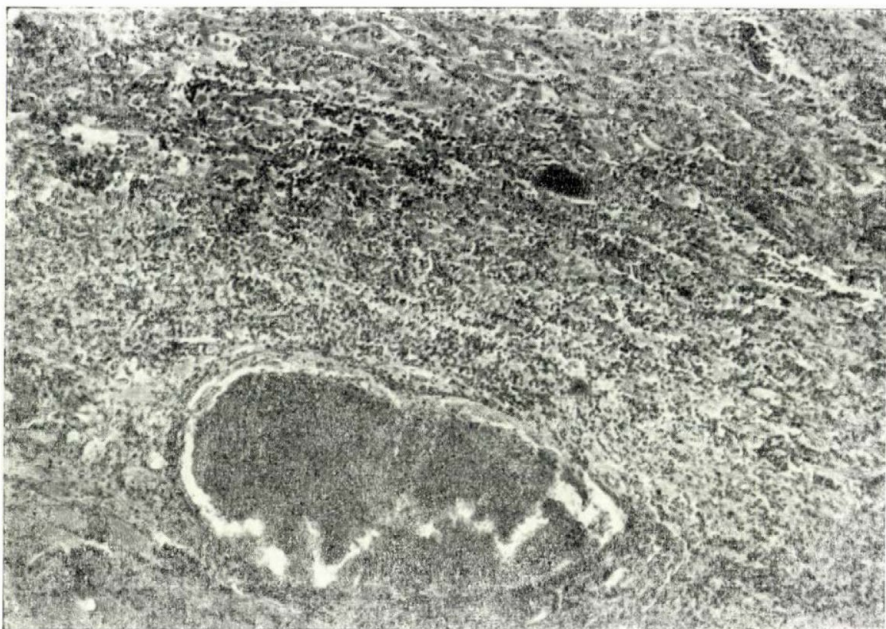


Fig. 3. Local Shwartzman phenomenon 24 hours after the eliciting injection. Thrombus-like obliteration of vessels; round cell and leucocytic infiltration; faint staining; necrosis. Haematoxylin-eosin, $\times 100$



Fig. 4. Local Shwartzman phenomenon 24 hours after the eliciting injection. Treatment with gold salt. Oedema in the cutis and subcutis, round cell and leucocytic infiltration, capillary stasis; no signs of necrosis. Haematoxylin-eosin, $\times 100$

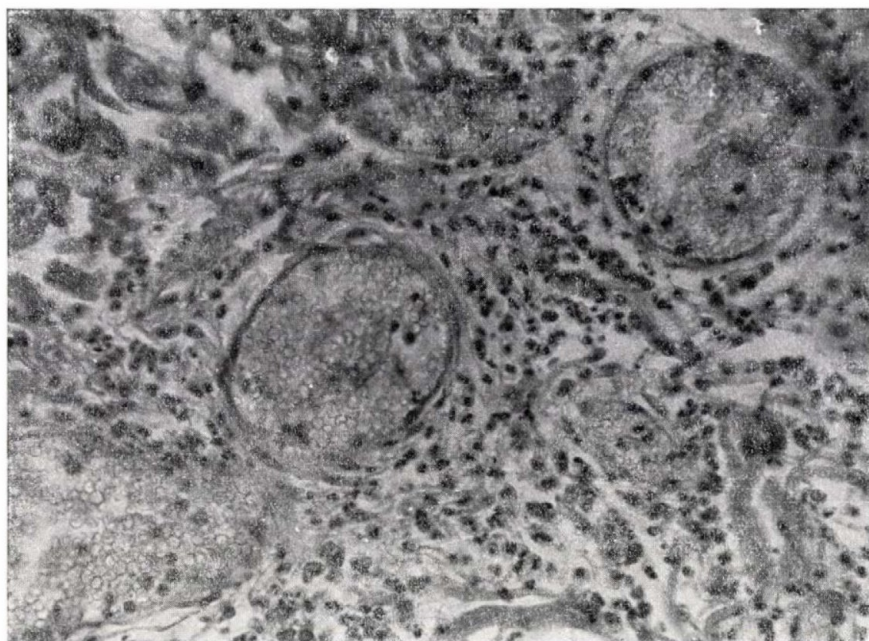


Fig. 5. Local Schwartzman phenomenon 24 hours after the eliciting injection. Treatment with gold salt. Stasis and leucocyte accumulation in vessels, precapillary oedema, round cell and leucocytic infiltration. Haematoxylin-eosin, $\times 480$

membrane. Hypothermia inhibited the development of both endotoxin and leucocyte homogenate-induced Schwartzman phenomenon in rabbits [7, 8]; in hypothermia the endotoxin is much less able to "release" lysosomal enzymes than in normothermia [9].

Inhibition of the Schwartzman phenomenon by gold salts may be explained in two different manners. The experiments of PERSELLIN and ZIFF [3] and LÉVAI *et al.* [5] indicate that the gold salt accumulates in the lysosomes. Gold may act as a stabilizer of the lysosomal membrane and may accordingly inhibit the release of enzymes. This hypothesis should be confirmed experimentally. Recent observations [3, 4, 10] indicate that gold compounds considerably inhibit SH enzymes and, consequently, the effect of lysosomal enzymes. THOMAS and STETSON [11] showed that SH enzymes play an important part in the production of lesions developing in the Schwartzman phenomenon, and cysteine has been found to enhance the development and intensity of the phenomenon [12].

In view of the above findings it may be concluded that locally administered gold salts act primarily on the lysosomes or on their enzymes. On local administration, gold salts are effective in relatively small amounts. In systemic treatment, however, the drug should be administered in larger doses and for prolonged periods of time.

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BIOCHEMICAL AND CULTURAL CHARACTERS OF SEROLOGICALLY GROUPED PSEUDOMONAS AERUGINOSA STRAINS

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Summary. The characters of 233 *Ps. aeruginosa* strains belonging to 23 different serological groups have been described. The strains had been isolated from a wide variety of sources and were selected so as to represent the average incidence of serological units.

According to the results and other authors' comparable data *Ps. aeruginosa* is characterized as follows. *Acid production in media containing slight amounts of complex nitrogenous substances.* Adonitol, dulcitol, m-inositol, inulin, lactose, D-maltose, raffinose, L-rhamnose, salicin, D-sorbitol, sucrose 100% negative. D-arabinose 99.1, L-arabinose 98.4, D-galactose 97.4, D-glucose (oxidative) 98.6, D-mannose 99.5, D-xylose 98.9% positive. Part of the strains produced acid from D-trehalose and L-xylose; repeated examinations with these substances yielded discrepant results in 24.4 and 24.0%, respectively. Most strains produce slight amounts of acid detectable only by delicate methods from D-fructose, glycerol and D-mannitol. *Growth in defined ammonium medium.* Adonitol, D-arabinose, L-arabinose, dulcitol, D-galactose, m-inositol, inulin, lactose, D-maltose, D-mannose, raffinose, L-rhamnose, salicin, D-sorbitol, sucrose, D-tartrate, D-xylose, L-xylose 100% negative. Acetate 99.8, benzoate 99.7, citrate 99.9, ethanol 99.7, D-fructose 99.5, D-glucose 99.7, glycerol 99.7, lactate 99.8, malonate 99.7, D-mannitol 98.7, propionate 99.6, succinate 99.7% positive. Variable reactions were observed in D-trehalose; growth in methanol was generally late and weak. *Other carbohydrate reactions.* Aesculin and starch hydrolysis, methyl red and Voges—Proskauer test 100% negative. *Nitrogenous substances.* Arginine dihydrolase 100% positive. Lysine and ornithine decarboxylase and tryptophan deaminase 100% negative. Gelatin liquefaction 99.1, lipolytic activity 99.3, nitrate reduction 100, utilization of urea as nitrogen source 99.7% positive. Indole production 100% negative. *Other characters.* Hydrogen sulphide production from thiosulphate 100% negative. Catalase, oxidase 100, haemolysin production 99.8% positive. Fluorescent pigment production 98.8, pyocyanin production 86.4, motility 99.5, growth at 41—42°C 99.9, growth at 4—5°C 0.2% positive. All strains multiplied in peptone water containing brilliant green, cetylpyridinium bromide, potassium cyanide, merthiolate, sodium chloride, sodium taurocholate and triphenyltetrazolium chloride at concentrations inhibiting the growth of many other bacteria. All strains grew on brilliant green, desoxycholate citrate, bismuth sulphite and eosin methylene blue agar.

It has been concluded that, disregarding pyocyanin production, acid production from D-trehalose and L-xylose and D-trehalose utilization shown to vary frequently within the same strain, *Ps. aeruginosa* is a homogeneous species the serological units of which practically cannot be subdivided into biotypes by the usual tests.

Recent studies on the classification of bacteria have attempted to define taxonomic units by determining the percentage of isolates possessing positive or negative test characters. In this manner the taxonomic importance of various properties may be considered and the data can be used in the identification of atypical strains.

Biochemical and cultural differences between members of large taxonomic units allow the classification of strains into smaller units as subgroups or biotypes. It has been shown in serologically well-defined bacteria that

biochemical and cultural characters are frequently closely associated with serological properties. In other organisms the serological units may be subdivided on the basis of biochemical differences.

The purpose of the present study was to examine the biochemical and cultural characters of serologically defined *Pseudomonas aeruginosa* strains isolated from a variety of sources. Another aim was to compare the results with other authors' data and to compile the percentage distribution of strains for each important biochemical and cultural character.

Materials and methods

Strains. From the author's collection consisting of 2197 *Ps. aeruginosa* strains isolated in the years 1958–1965 [32] 233 cultures were selected so as to represent the average incidence of serological groups. The strains originated from various hospitals, communities and other sources. The distribution of the strains according to serological groups and sources is presented in Table I.

The cultures were stored at room temperature in agar stab cultures stoppered with cork sterilized by heating in paraffin. Subcultures were made at about 1 1/2 year intervals. The medium used for the maintenance of the cultures was as follows: agar, 14 g; beef extract (Oxoid), 10 g; Proteose peptone (Oxoid), 10 g; distilled water, 1000 ml; pH 7.4. More important strains were maintained also by freeze-drying.

Inoculation of media. Unless otherwise indicated the tests were carried out in 2 to 3 ml portions of media distributed into 100 × 11 mm tubes. Seeding was performed from agar slants incubated at 37°C overnight then at room temperature for 3 days. Liquid media were inoculated by immersing a small amount of solid culture by means of a straight wire. Solid media were seeded by stab inoculation. Agar plates were inoculated with a loopful of 18 to 20 hour broth culture.

Sugars, alcohols, organic acids and allied compounds. The following substances were used: sodium acetate, adonitol (Merck), aesculin (BDH), D-arabinose (BDH), L-arabinose (Fluka), sodium benzoate, sodium citrate, dulcitol (Merck), ethanol, sodium formate (Merck), D-fructose (Light), D-galactose (Light), glycerol, D-glucose, m-inositol (Merck), inulin (Scheering—Kahlbaum), lactose, lactic acid, malonic acid (BDH), D-maltose, D-mannitol, D-mannose, methanol, sodium oxalate, propionic acid, D-raffinose (Light), L-rhamnose (Light), salicin (BDH), D-sorbitol (Light), starch, succinic acid, sucrose, D-tartrate (Merck), D-trehalose (BDH), D-xylose, L-xylose (Light). Substances without brand marking were prepared by Hungarian firms (Finomvegyszer or Reanal, Budapest).

(a) **Acid production in 1% peptone water.** Bacto peptone (Difco) 10 g; NaCl, 5 g; distilled water, 1000 ml; Andrade indicator, 10 ml; substrate, 10 g; pH 7.2; Seitz filtration. The tubes were incubated at 37°C for 14 days. Differentiation of oxidative and fermentative glucose breakdown was performed in two tubes of peptone water. One of them was incubated aerobically, the duplicate was sealed with liquid paraffin after boiling and seeding.

(b) **Acid production in 0.1% peptone water.** The above medium was prepared with 0.1% peptone.

(c) **Utilization of sugars, alcohols and allied substances as sole sources of carbon.** The medium described by SELEEN and STARK [51] was prepared with 1% substrate and sterilized by Seitz filtration. The tubes were incubated at 37°C for 14 days. Tubes showing definite growth were recorded as positive.

(d) **Utilization of organic acids as sole sources of carbon.** The medium of SELEEN and STARK [51] was prepared with 0.3% substrate and sterilized by Seitz filtration. The media were incubated and read as described above for sugar and alcohol utilization tests.

(e) **Aesculin hydrolysis.** The usual method was employed [5]. Incubation lasted for 14 days at 37°C.

(f) **Starch hydrolysis.** Beef extract agar, 1000 ml; potato starch, 2 g. The plates were incubated for 6 days at 37°C then flooded with Lugol solution.

(g) **Methyl red test.** Bacto peptone (Difco), 7 g; K_2HPO_4 , 5 g; glucose, 5 g; distilled water, 1000 ml; pH 7.5; autoclaving at 110°C for 20 minutes. Incubation lasted for 4 days at 37°C. The results were read as described in reference 5.

Table I

Distribution of the examined Ps. aeruginosa strains according to serological groups and sources

O antigen*	Blood and CSF	Pus and wounds	Urine	Faeces	Other human material	Water and sewage	Total
1	2	3	1	1	1	1	9
2	—	—	1	4	1	3	9
3a, 3b	1	7	1	3	3	1	16
3c	1	7	2	—	1	—	11
3a, 3d	2	6	2	2	2	1	15
3a, 3d, 3e	—	5	2	1	1	1	10
3d, 3f	2	4	1	2	2	—	11
4a, 4b	—	5	1	3	1	1	11
4a, 4c	—	3	1	4	1	2	11
4a, 4d	1	7	—	3	3	2	16
5a, 5b, 5c	—	4	1	1	—	1	7
5a, 5b, 5d	—	7	1	1	—	2	11
5a, 5d	—	9	1	2	2	2	16
6	—	5	—	5	4	2	16
7a, 7b	—	8	1	3	3	1	16
7a, 7c	—	1	—	—	4	—	5
8	1	—	2	1	—	—	4
9	—	2	—	1	1	—	4
10a	—	5	1	1	—	4	11
10a, 10b	—	1	—	4	—	1	6
11	—	6	—	2	—	3	11
12	—	—	—	—	4	2	6
13	—	—	—	—	—	1	1
Total	10	95	19	44	34	31	233

* LÁNYI's antigenic schema [32].

(h) *Voges—Proskauer test*. The strains were cultured in methyl red medium at 37°C for 4 days then equal portions of 40% KOH and some grains of creatine were added to the tubes. Positive reaction appeared after a few hours standing as pink coloration.

Nitrogenous substances. (a) *Amino acids*. Lysine and ornithine decarboxylase and arginine dihydrolase were shown as described by MØLLER [41] except that Bacto-Tryptone (Difco) was substituted for Orthana peptone. The substrates were DL-lysine dihydrochloride (Schuchardt), L-ornithine monohydrochloride (Reanal) and L-arginine hydrochloride (Reanal). The cultures were incubated at 37°C for 6 days. Positive reaction was indicated by the appearance of a violet colour; negative tubes were characterized by slate-grey colour instead of the yellow colour shown by *Enterobacteriaceae*.

Tryptophan deaminase was tested as described by LÁNYI and ÁDÁM [33].

(b) *Indole production*. Bacto peptone (Difco), 10 g; NaCl, 5 g; distilled water, 1000 ml; pH 7.2. Incubation lasted for 4 days at 37°C. The results were read as described by Kovács [30].

(c) *Lipolytic activity.* The following technique based on the methods of COLLINS and HAMMER [4], DOWSON [8] and RHODES [44] was used. Basal medium: $(\text{NH}_4)_2\text{HPO}_4$, 1 g; KCl, 0.2 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; beef extract (Oxoid), 3 g; agar, 18 g; distilled water, 1000 ml; pH 7.8. To 100 ml hot basal medium 8 ml 0.3% alcoholic solution of commercial grade Nile blue sulphate and 20 ml oil emulsion were added. Oil emulsion: acacia, 2.5 g; distilled water, 100 ml; castor oil, 25 ml; the emulsion was sterilized at 100°C for 10 minutes, then homogenized in a Turmix blender. The plates were incubated at 37°C for 24 hours. Under and around the growth of lipase-producing bacteria the red oil globules turned red (free fatty acid).

(d) *Nitrate reduction.* Beef extract (Oxoid), 3 g; Bacto peptone (Difco), 5 g; KNO_3 , 1 g; distilled water, 1000 ml; pH 7.4. The medium was distributed at 5 ml portions into test tubes. Incubation lasted for 4 days at 37°C then Griess—Ilosvay reagent was added and finally the zinc dust test was performed.

(e) *Proteolytic activity. Gelatin liquefaction* was examined by LAUTROP's method [35]. The denatured gelatin columns were placed into 5 ml 18–20 hour broth cultures and incubated at 37°C for 34 days. Testing of strains which showed no gelatin hydrolysis after 14 days was repeated in dense bacterial suspensions harvested from agar slants. *Digestion of serum.* Loeffler slopes (pH 7.4 beef extract-peptone broth, 350 ml; glucose, 3.5 g; ox serum, 650 ml) were seeded and incubated at 37°C for 28 days.

(f) *Hydrolysis and utilization as nitrogen source of urea.* NaCl, 5 g; KH_2PO_4 , 2 g; 0.2% phenol red solution, 6 ml; distilled water, 1000 ml; urea, 20 g; glucose, 1 g; pH 6.9. The medium was sterilized by Seitz filtration. Positive reaction was indicated by red coloration and growth.

Other characters. (a) *Haemolysin production.* Blood agar: peptone (Richter), 10 g; agar, 14–18 g; NaCl, 3 g; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 4 g; baker's yeast, 40 g; tap water, 1000 ml; pH 7.4; defibrinated ox blood, 50 ml. The plates were incubated at 37°C for 24 hours then at room temperature for another day.

(b) *Catalase activity* was examined by the slide test: a loopful of agar slant culture was mixed into one drop of concentrated H_2O_2 solution. Doubtful reactions were repeated by adding 0.5 ml concentrated H_2O_2 to 5 ml 18–20 hour broth culture.

(c) *Hydrogen sulphide production.* Stab inoculations were made into the standard thiosulphate agar described by RAUSS and VÖRÖS [43]. Incubation lasted for 4 days at 37°C.

(d) *Oxidase production* was tested by KOVÁCS's method [31]. Production of a purple colour within 20 seconds on filter paper strips impregnated with the reagent was recorded as positive.

(e) *Pigment production.* Pyocyanin production was shown by the use of "medium A" described by KING *et al.* [22]. After incubating the slants for 4 days at 37°C 4 to 5 ml chloroform were added. The tubes were left to stand for 2 to 3 hours then read. *Fluorescein* was detected in fluid "medium B" of KING *et al.* [22]. The medium was distributed at 5 to 6 ml amounts into test tubes and incubated after seeding for 3 days at 37°C then for 4 days at room temperature. Readings were performed at 365 m μ wave-band UV light in a Spekker Model H764 fluorimeter supplied with mercury vapour lamp and H556 filter. Fluorescein positive cultures showed bright green fluorescence; strains not producing this pigment were characterized by faint bluish fluorescence.

(f) *Motility.* Broth cultures incubated for 18 to 20 hours at 37°C were examined in a darkfield microscope with the low-power objective. If the majority of cells showed no active motility, the examination was repeated after U-tube passage.

The effect on growth of incubation temperature and inhibitory substances. (a) *Growth temperature.* Beef extract broth was distributed at 5 ml amounts into 4 series of test tubes. The media were inoculated with a straight wire and incubated at 5, 20, 37 and 42°C. Reading of 5°C tubes was performed after one week; tubes incubated at 20, 37 and 42°C showed good growth after 24 hours.

(b) *Inhibitory substances.* Each substance was incorporated in the following basal medium: Proteose peptone (Difco), 3 g; NaCl, 5 g; KH_2PO_4 , 0.225 g; K_2HPO_4 , 5.64 g; distilled water, 1000 ml; pH 7.6; autoclaving at 121°C for 20 minutes. The concentrations of the inhibitory substances were: brilliant green, 10 $\mu\text{g}/\text{ml}$; cetylpyridinium bromide, 10 $\mu\text{g}/\text{ml}$; potassium cyanide, 75 $\mu\text{g}/\text{ml}$; merthiolate, 0.25 $\mu\text{g}/\text{ml}$; sodium chloride, 5.4%; sodium taurocholate, 5%; triphenyltetrazolium chloride, 1%. Tubes containing KCN medium were stoppered with cork heated in paraffin. Incubation lasted for 4 days at 37°C. Tubes showing definite growth were recorded as positive.

Growth on some selective and differential media. Plated media specified for the Hungarian Public Health Laboratory Network were inoculated with loopful amounts of 18 to 20 hour broth cultures and incubated at 37°C for 20 to 22 hours then at room temperature for one day.

(a) *Bismuth sulphite agar*. Basal medium: pork extract, 16 g; tryptic digest of casein, 8 g; agar, 15–18 g; tap water, 1000 ml; pH 7.2. Basal mixture: glucose, 50 g; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 50 g; bismuth ammonium citrate, 50 g; Na_2SO_3 , 25 g; ferrous ammonium sulphate, 6.5 g; brilliant green, 0.25 g; distilled water to 1075 ml. To 5 volumes of basal medium 1 volume of basal mixture was added.

(b) *Brilliant green agar*. Tryptic digest of casein, 6.6 g; peptone (Richter), 1.1 g; yeast cell supernatant, 15 ml; yeast cells, 0.75 ml; NaCl, 5 g; agar, 15–17 g; tap water, 1000 ml; pH 7.4; Andrade indicator, 30 ml; lactose, 10 g; sucrose, 1 g; glucose, 0.5 g; 0.1% aqueous brilliant green solution, 4 ml.

(c) *Desoxycholate citrate agar*. Pork extract, 15 g; peptone (Richter), 1 g; tryptic digest of casein, 9 g; agar, 15–18 g; lactose, 10 g; sodium citrate, 23 g; sodium desoxycholate, 3 g; lead acetate, 0.1 g; ferrous ammonium sulphate, 1 g; 1% aqueous neutral red solution, 1 ml.

(d) *Eosin methylene blue agar*. Peptone (Richter), 10 g; yeast supernatant (1000 g baker's yeast + 1200 ml water autoclaved at 121°C for 30 minutes), 15 ml; NaCl, 3 g; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 4 g; agar, 14–18 g; tap water, 1000 ml; pH 7.4; lactose, 10 g; Mavekal (anionic detergent [10]), 10 ml; eosin, 0.4 g; methylene blue, 0.075 g.

Results

1. *Sugars, alcohols, organic acids and allied substances*. Table II presents data for acid production in 0.1% peptone water and utilization as carbon source in chemically defined medium. Acid production was examined also in the presence of 1% peptone. In the latter medium most strains produced acid from L-arabinose, D-galactose, D-glucose and D-xylose. In 0.1% peptone water the overwhelming majority of strains produced acid from D-arabinose and D-mannose in addition to the above substances. Variable reactions were obtained in L-xylose and D-trehalose. Neither growth nor acid production was detected in glucose peptone water incubated anaerobically.

The results indicated that 0.1% peptone water was more suitable for the detection of acid production than the classical medium. In the former acid production appeared earlier with all substances except glucose and reversion to neutral reaction occurred much less frequently than in the presence of 1% peptone. Preliminary experiments indicated that 0.2 to 0.05% peptone concentration and Andrade indicator gave the best results.

Differences between the isolates in acid production from D-trehalose and L-xylose suggested that the two reactions might be useful for classification purposes. Repeated examination of all strains (D-trehalose II and L-xylose II in Table II) as well as multiple tube experiments with some strains, however, failed to confirm this expectation. The results are detailed under "Distribution of biochemical characters according to serological groups".

In comparing acid production in the presence of peptone and growth ability in defined medium the results differed for certain substrates. Most strains produced acid from D-arabinose, L-arabinose, D-galactose, D-mannose and D-xylose but were unable to use these substances as sole sources of carbon. In defined medium with D-arabinose and D-galactose some strains showed weak growth at the end of the 2-week incubation period. These reactions were recorded as negative.

Table II

Examination of sugars, alcohols and allied substances. Acid production in peptone water and growth in defined ammonium medium

Number of strains, 233

	Acid production in 0.1% peptone water				Growth in defined ammonium medium			
	1—2 days	3—6 days	7—14 days	negative	1—2 days	3—6 days	7—14 days	negative
Adonitol	—	—	—	233	—	—	—	233
D-Arabinose	77	135	18	3	—	—	—	233
L-Arabinose	82	144	5	2	—	—	—	233
Dulcitol	—	—	—	233	—	—	—	233
Ethanol	.	.	.	.	187	45	—	1
D-Fructose	—	—	—	233	25	207	—	1
D-Galactose	97	130	4	2	—	—	—	233
Glycerol	—	—	—	233	92	126	14	1
D-Glucose	191	35	5	2	226	5	1	1
m-Inositol	—	—	—	233	—	—	—	233
Inulin	—	—	—	233	—	—	—	233
Lactose	—	—	—	233	—	—	—	233
D-Maltose	—	—	—	233	—	—	—	233
D-Mannitol	—	—	—	233	115	91	22	5
D-Mannose	97	122	12	2	—	—	—	233
Methanol	.	.	.	.	—	225 ×	7 ×	1
Raffinose	—	—	—	233	—	—	—	233
L-Rhamnose	—	—	—	233	—	—	—	233
Salicin	—	—	—	233	—	—	—	233
D-Sorbitol	—	—	—	233	—	—	—	233
Sucrose	—	—	—	233	—	—	—	233
D-Trehalose I.	—	5	103	125	6	151	12	64
D-Trehalose II.	—	—	152	81	.	.	.	.
D-Xylose	230	1	2	—	—	—	—	233
L-Xylose I.	81	46	18	88	—	—	—	233
L-Xylose II.	—	73	62	98	.	.	.	.

× = Weak growth

. = Not performed

The isolates considerably differed in the time of the appearance of positive reactions. Repeated experiments have shown that earlier or later acid production or utilization cannot be used as a differential criterion: the time elapsing until positivity had become evident considerably varied even within one strain.

Table III
Examination of organic acids. Growth in defined ammonium medium
 Number of strains, 233

	Growth in defined ammonium medium			
	1—2 days	3—6 days	7—14 days	negative
Acetate	229	2	1	1
Benzoate	174	41	17	1
Citrate	232	—	—	1
Formate	—	—	—	233
Lactate	231	1	—	1
Malonate	231	—	1	1
Oxalate	—	—	—	233
Propionate	8	199	25	1
Succinate	232	—	—	1
D-Tartrate	—	—	—	233

Table III shows the utilization of organic acids in defined medium. All strains but one grew abundantly in acetate, benzoate, citrate, lactate, malonate, propionate and succinate. Strain 171005 which was unable to grow at the expense of these substances failed to utilize carbohydrates and alcohols as carbon source and urea as nitrogen source. In the presence of peptone this strain behaved as typical *Ps. aeruginosa* cultures do.

Other reactions associated with carbohydrate metabolism (aesculin and starch hydrolysis, methyl red and Voges—Proskauer reaction) were negative for all strains.

2. *Nitrogenous substances.* Among amino acid tests used in bacteriology *arginine dihydrolase* was strongly positive for 231 strains; 2 strains developed positive reaction between the third and sixth day. All strains were *lysine* and *ornithine decarboxylase* and *tryptophan deaminase* negative.

None of the cultures produced *indole*. All strains exerted definite *lipolytic activity*. *Nitrate* was reduced beyond nitrite by all strains. After 5 days incubation neither nitrate nor nitrite was present in 229 cultures; the remaining 4 cultures showed traces of nitrite still after 5 to 14 days.

Proteolytic activity. Denatured gelatin in broth cultures was liquefied by 194 strains in 2 days, by 16 strains in 14 days; 23 strains gave positive reactions on further incubation (up to 34 days). The proteolytic activity in the slow liquefiers could be accelerated by placing the gelatin columns into dense suspensions harvested from agar cultures.

Loeffler's serum was liquefied by all except 7 strains within 14 days. The non-liquefying strains caused also a more or less definite softening of the medium.

In defined medium containing *urea* as a sole source of nitrogen 217 strains multiplied and caused alkaline reaction within 2 days. Alkalinity developed less rapidly in 15 cultures. Some of these turned at first acid (acid production from glucose exceeded alkali production from urea). One isolate, ammonium deficient strain 171005, failed to grow in defined urea medium.

3. *Other characters.* All strains except one (a weak haemolysin producer) showed complete *haemolysis*. The *catalase* test was generally strongly positive, only one strain gave a weak reaction. This (strain 170009 = Ps 217, type strain for O antigen subgroup 4a, 4c) was the only non-motile culture among the 233 strains examined.

The *oxidase* test was positive within a few seconds for 296, within 20 seconds for 37 strains.

In fluid "King B" medium after 3 days incubation at 37°C 218 strains produced *fluorescin*. On further incubation at room temperature for 4 days 12 cultures developed fluorescence. Thus only 3 out of the 233 isolates were fluorescein negative (these cultures failed to show fluorescence not only in fluid but also on solid "King B" medium).

On "King A" medium 187 strains produced *pyocyanin*. All strains but one were *actively motile*.

4. *The effect of incubation temperature and of inhibitory substances: growth on selective media.* In broth all strains grew well after one day at 20, 37 and 42°C, but failed to multiply within one week at 5°C.

All strains grew in brilliant green, cetylpyridinium bromide, potassium cyanide, merthiolate, sodium chloride, sodium taurocholate and triphenyl-tetrazolium chloride peptone water indicated under "Materials and methods". Brilliant green, merthiolate, sodium chloride and triphenyltetrazolium chloride at the given concentration and in the specified medium inhibited the growth of many of the tested *Enterobacteriaceae* strains. In cetylpyridinium bromide none of these organisms was able to multiply. In potassium cyanide medium used in the identification of *Enterobacteriaceae* all *Ps. aeruginosa* strains grew well. Five per cent sodium taurocholate was tolerated by *Ps. aeruginosa* and *Enterobacteriaceae* strains but not by bacteria belonging to many other families.

All strains grew on selective media commonly used in the diagnosis of *Enterobacteriaceae*. Colonies on *bismuth sulphite agar* were mostly uniform, minute or small, greenish-white with smooth surface. Colonies on *brilliant green agar* were somewhat smaller than on nutrient agar; different colonial forms of *Ps. aeruginosa* [11, 13, 63, 68] could well be distinguished. Colonies on *desoxycholate citrate agar* were mostly uniform, medium or large sized, convex, mucous dark pink in colour with glistening surface. Colonies on *eosin methylene blue agar* were smaller than on brilliant green agar; although poly-

Table IV
Atypical strains

Designation	O antigen*	Atypical property
170009	4a, 4c	Non-motile, exerts weak catalase activity
170018	9	Fluorescin negative
171005	1	Fails to utilize ammonium and urea nitrogen
171107	3a, 3b	Fails to produce acid from D-arabinose (produces weakly D-arabinose positive colonial variants)
171124	3c	Fluorescin negative
171126	3c	Fluorescin negative
171134	3c	Fails to utilize D-mannitol (produces D-mannitol-utilizing colonial variants)
171156	3a, 3d	Fails to produce acid from L-arabinose, D-glucose, D-galactose and D-mannose (produces weakly L-arabinose positive colonial variants)
171167	3a, 3d	Fails to utilize D-mannitol
171188	3a, 3d, 3e	Fails to utilize D-mannitol
171299	5a, 5b, 5c	Fails to utilize D-mannitol
171304	5a, 5b, 5c	Fails to produce acid from D-arabinose (produces weakly D-arabinose positive colonial variants)
171453	9	Fails to produce acid from L-arabinose, D-glucose, D-galactose and D-mannose (produces weakly L-arabinose positive colonial variants)
171518	12	Fails to produce acid from D-arabinose

* LÁNYI's antigenic schema [32].

morphism appeared, the colonial types could not be distinguished so clearly as on nutrient or brilliant green agar.

5. *Distribution of biochemical characters according to serological groups.* The results suggested that acid production from D-trehalose and L-xylose might be suitable for differentiating the strains. As seen in Table II experiments repeated for these substances showed significant discrepancies from the first series of tests if the total number of strains was considered. For individual strains the following results were obtained. D-Trehalose: the results of the two examinations were identical for 176 strains (76 positive, 100 negative); discrepant results (first examination positive, second examination negative or *vice versa*) were shown by 57 strains. L-Xylose: 177 strains gave identical (114 positive, 63 negative), 56 strains discrepant results. Many cultures recorded as negative showed indefinite, weak acid reaction on the 14th day of incubation.

Pyocyanin production has been described by several authors as a property variable within one strain and there are data that in the course of laboratory maintenance the ability of strains to produce this pigment is frequently lost. Cultures used in the present study were examined for pyocyanin production twice: shortly after isolation and in this work. Most of the 46 pyocyanin negative strains encountered had been isolated in the years 1958—1961 and were subcultured 5 to 8 times since. Only 10 out of the 46 strains produced pyocyanin originally, 36 were negative also at the first testing. That is, only 10 out of the 197 originally pyocyanin-producing isolates had lost this property (5.1%).

No significant difference was revealed between serological units as to the distribution of D-trehalose, L-xylose and pyocyanin positive and negative strains. Other properties examined in this study could not be used for differentiation purposes. As shown in Table IV not more than 14 atypical cultures were encountered. Multiple-tube experiments performed with the atypical strains indicated that some of them tended to split off variants reacting in certain tests as typical *Ps. aeruginosa* cultures (see Table IV).

Discussion

Although the biochemical properties of *Ps. aeruginosa* have been examined by several authors, the results have not been included in bacteriological handbooks and even BERGEY's Manual [2] defines some characters of *Ps. aeruginosa* incorrectly. This may perhaps be explained by the fact that several discrepant results have been described. In the following, the present results will be compared to other authors' data and an attempt will be made at specifying the methods most adequate for the characterization of *Ps. aeruginosa*.

1. *Sugars, alcohols, organic acids and allied substances.* Most discrepancies between various data have been noted in this group of tests. Methods used for the detection of acid production by *Ps. aeruginosa* may be divided into three main groups according to the basal medium. 1. Classical media: SANDIFORD [47] and LYSENKO [40]. 2. Test media poor in complex nitrogenous substances: BENDER and LEVINE [1], HUGH and LEIFSON [19], SIMON [55], VÉRON and CHATELAIN [62], ZIERDT and SCHMIDT [68] and JESSEN [21]. 3. ELROD and BRAUN [9], STEIN *et al.* [58], SELEEN and STARK [51], SALVIN and LEWIS [46], LIU [36] and LYSENKO [40] examined acid production in defined media containing inorganic nitrogenous compounds and compared the results with tests in peptone media. SEARS and GOURLEY [50], DE BORD [6] and LIU [36] showed by quantitative examinations that in *Ps. aeruginosa* cultures the small amount of acid produced

Table V

Summary of important characters of *Ps. aeruginosa*
Acid production from sugars, alcohols and allied substances

	Present findings		Comparable data of other authors*		Summarized data		
	positive	negative	positive	negative	No. of strains	per cent positive	per cent negative
Adonitol	—	233	—	403 [20, 21, 36] 4 × [21]	640	—	100.0
D-Arabinose	230	3	498 [21, 40, 55, 62]	4 × [21]	735	99.1	0.9
L-Arabinose	231	2	524 [21, 36, 40, 55]	10 [55]	767	98.4	1.6
Dulcitol	—	233	—	569 [20, 21, 36, 46, 55, 64]	802	—	100.0
D-Fructose	—	233	87 [20, 36, 40]	45 [20, 46]	Different according to method		
D-Galactose	231	2	211 [20, 36, 46, 55, 62, 68]	10 [55]	454	97.4	2.6
Glycerol	—	233	540 [21, 36, 40, 46, 51, 62, 64]	30 [9, 21, 62]	Different according to method		
D-Glucose	231	2	1133 [9, 12, 20, 21, 36, 40, 46, 47, 51, 55, 61, 62, 64, 68]	17 [9, 46, 55, 61, 62]	1383	98.6	1.4
m-Inositol	—	233	—	467 [9, 20, 21, 36, 46]	700	—	100.0
Inulin	—	233	—	108 [36, 46, 64]	341	—	100.0
Lactose	—	233	—	968 [12, 20, 21, 40, 46, 47, 51, 55, 62, 64, 68] 11 × [20, 21]	1212	—	100.0
D-Maltose	—	233	—	599 [9, 20, 21, 36, 46, 55, 64, 68] 1 × [21]	833	—	100.0
D-Mannitol	—	233	446 [20, 21, 36, 40, 62, 68]	85 [9, 20, 21, 46, 62]	Different according to method		
D-Mannose	231	2	144 [20, 36, 40, 46, 68]	—	377	99.5	0.5
Raffinose	—	233	—	586 [9, 20, 21, 36, 46, 55, 64]	819	—	100.0
L-Rhamnose	—	233	4 [20, 62]	180 [20, 21, 36, 46, 62] 280 × [21]	697	—	100.0**
Salicin	—	233	—	543 [9, 20, 21, 36, 55, 64]	776	—	100.0
D-Sorbitol	—	233	—	450 [20, 21, 36, 46]	683	—	100.0
Sucrose	—	233	—	1041 [9, 12, 20, 21, 36, 40, 46, 47, 51, 55, 62, 64, 68]	1274	—	100.0
D-Trehalose	I: 108 II: 152	125 81	145 [20, 21, 55]	217 [20, 21, 46, 55] 142 × [21]	Not stable		
D-Xylose	233	—	638 [9, 20, 21, 36, 40, 46, 55, 62, 64, 68]	10 [55]	881	98.9	1.1
L-Xylose	I: 145 II: 135	88 98	—	—	Not stable		

* = Number of *Ps. aeruginosa* strains; bracketed figures indicate references.

** = Weak, delayed acid production observed frequently in sensitive media (OF, defined).

× = Weak, delayed acid production.

Table VI

Summary of important characters of *Ps. aeruginosa*
Growth with sugars, alcohols, organic acids and allied substances in defined ammonium medium

	Present findings		Comparable data of other authors*		Summarized data		
	positive	negative	positive	negative	No. of strains	per cent positive	per cent negative
Adonitol	—	233	—	139 [36, 42, 56]	372	—	100.0
D-Arabinose	—	233	—	29 [56]; 65 × [42]	327	—	100.0**
L-Arabinose	—	233	—	74 [36, 56]	307	—	100.0
Dulcitol	—	233	—	115 [36, 42, 44]	348	—	100.0
Ethanol	232	1	162 [21, 40, 56]	—	395	99.7	0.3
D-Fructose	232	1	138 [36, 42, 56]	1 [56]	372	99.5	0.5
D-Galactose	—	233	—	29 [56]; 97 × [21]	359	—	100.0**
Glycerol	232	1	144 [36, 42, 44, 56]	—	377	99.7	0.3
D-Glucose	232	1	144 [36, 42, 44, 56]	—	377	99.7	0.3
m-Inositol	—	233	—	139 [36, 42, 56]	372	—	100.0
Inulin	—	233	—	45 [36]	278	—	100.0
Lactose	—	233	—	139 [36, 42, 56]	372	—	100.0
D-Maltose	—	233	—	139 [36, 42, 56]	372	—	100.0
D-Mannitol	228	5	139 [36, 42, 56]	—	372	98.7	1.3
D-Mannose	—	233	—	74 [36, 56]	307	—	100.0
Methanol	—	1 232 ×	—	—	Weak, delayed utilization		
Raffinose	—	233	—	110 [36, 42]	343	—	100.0
L-Rhamnose	—	233	—	139 [36, 42, 56]	372	—	100.0
Salicin	—	233	—	144 [36, 42, 44, 56]	377	—	100.0
D-Sorbitol	—	233	—	139 [36, 42, 56]	372	—	100.0
Sucrose	—	233	—	139 [36, 42, 56]	372	—	100.0
D-Trehalose	169	64	115 [36, 42, 44]	29 [56]	Not stable		
D-Xylose	—	233	—	74 [36, 56]	307	—	100.0
L-Xylose	—	233	—	—	233	—	100.0
Acetate	232	1	208 [21, 40, 44, 51, 56]	—	441	99.8	0.2
Benzoate	232	1	65 [40, 56]	—	298	99.7	0.3
Citrate	232	1	524 [20, 21, 39, 40, 42, 44, 51, 56, 61, 64, 68]	—	757	99.9	0.1
Lactate	232	1	172 [21, 44, 51, 56]	—	405	99.8	0.2
Malonate	232	1	73 [20, 40, 56]	—	306	99.7	0.3
Propionate	232	1	—	—	233	99.6	0.4
Succinate	232	1	131 [20, 44, 56]	—	364	99.7	0.3
D-Tartrate	—	233	—	138 [20, 51]	371	—	100.0

Discrepant results: D-arabinose, 5+ [44]; L-arabinose, 70+ [42, 44]; D-fructose, 5+ [44]; D-galactose, 115+ [36, 42, 44]; m-inositol, 5+ [44]; inulin, 65+ [42]; lactose, 5+ [44]; D-maltose, 5+ [44]; D-mannose, 65+ [42]; raffinose, 5+ [44]; L-rhamnose, 5+ [44]; D-sorbitol, 5+ [44]; sucrose, 5+ [44]; D-xylose, 65+ [42].

* Number of *Ps. aeruginosa* strains; bracketed figures indicate references.

** Weak, delayed growth may occur.

× = Weak, delayed utilization.

Table VII

Summary of important characters of *Ps. aeruginosa*
Other properties

	Present findings		Comparable data of other authors*		Summarized data		
	positive	negative	positive	negative	No. of strains	per cent positive	per cent negative
Aesculin hydrolysis	—	233	—	70 [42, 44]	303	—	100.0
Gluconate test	—	233	259 [17, 20, 40, 42, 44, 56, 63, 68]	—	259	100.0	—
Starch hydrolysis	—	233	—	373 [21, 36, 40, 42, 46, 51, 56]	606	—	100.0
Methyl red	—	233	—	22 [20, 68]	255	—	100.0
Voges-Proskauer	—	233	—	68 [20, 21, 40, 68]	301	—	100.0
Arginine dihydro- lase	233	—	135 [3, 20, 40, 54, 68]	—	368	100.0	—
Lysine decarboxy- lase	—	233	—	67 [20, 40, 61]	300	—	100.0
Ornithine decar- boxylase	—	233	—	44 [20, 40]	277	—	100.0
Tryptophan de- aminase	—	233	—	—	233	—	100.0
Gelatin liquefac- tion	233	—	720 [9, 21, 39, 40, 42, 44, 46, 51, 56, 63, 64]	9 [39, 46, 64]	962	99.1	0.9
Indole production	—	233	—	826 [3, 9, 20, 21, 39, 40, 42, 46, 47, 51, 56, 64, 68]	1059	—	100.0
Lipolytic activity	233	—	181 [21, 40, 44, 56]	3 [21]	417	99.3	0.7
Nitrate reduction	233	—	669 [20, 21, 37, 39, 40, 42, 44, 51]	—	902	100.0	—
Urease activity in defined medium	232	1	106 [40, 42, 44]	—	339	99.7	0.3
H ₂ S from thio- sulphate	—	233	—	—	233	—	100.0
Catalase	233	—	463 [20, 21, 40, 42]	—	696	100.0	—
Oxidase (Kovács)	233	—	934 [21, 31, 40, 42, 56, 57]	—	1167	100.0	—
Oxidase (Gaby & Hadley)	—	233	389 [24, 26, 29, 48, 68]; 37 × [29]	—	426	100.0	—
Haemolysin	233	—	606 [21, 28, 39, 40, 42, 46]	2 [21, 28]	841	99.8	0.2
Fluorescein	230	3	866 [3, 21, 26, 39, 40, 42, 51, 61, 63]	10 [21, 61]	1109	98.8	1.2
Pyocyanin	187	46	916 [3, 9, 17, 18, 21, 26, 40, 42, 46, 51, 55, 56, 61]	128 [3, 9, 17, 18, 21, 26, 40, 42, 51, 56, 61]	1277	86.4	13.6
Motility	232	1	385 [18, 60, 68]	2 [18]	620	99.5	0.5
Growth at 41— 42 °C	233	—	1211 [3, 17, 18, 21, 26, 28, 39, 40, 42, 44, 46, 51, 56, 63, 64]	2 [21]	1446	99.9	0.1
Growth at 4—5 °C	—	233	2 [21]	548 [18, 21, 40, 46, 51, 56]	783	99.8	0.2

* Number of *Ps. aeruginosa* strains; bracketed figures indicate references.

× = Weak, delayed reaction.

from carbohydrates was frequently neutralized by alkali formed abundantly from peptone, the constant ingredient of media used in these tests. The experiments of SELEEN and STARK [51] indicated that testing at low peptone concentrations gave more readily interpretable results than examination in classical peptone water media. Several other authors came to the same conclusion.

In the present studies 0.1% peptone ensured the best results. Acid production from glucose appeared frequently earlier in 1% than in 0.1% peptone water, due to the fact that *Ps. aeruginosa* growing rapidly and abundantly in the former medium attacks glucose more readily than other carbohydrates. Acid production spectra in defined ammonium medium were generally similar to those obtained in 0.1% peptone water, but the amount of acid was small and the reactions were therefore indefinite and not easily interpretable. Similar observations were made by PULVERER and KORTH [42]. Of test media poor in peptone the oxidation-fermentation (OF) medium described by HUGH and LEIFSON [19] has widely been used for detecting acid production by *Ps. aeruginosa* (HUGH and LEIFSON [20], ZIERDT and SCHMIDT [68], JESSEN [21]). Our fluid medium was less sensitive than OF medium because the shift in the colour of Andrade indicator occurred at a lower pH than that of bromthymol blue and, in contrast to the semi-fluid OF medium, there was no local acid accumulation in it. Acid reactions in cultures producing pigment abundantly were more easily readable with Andrade than with bromthymol blue indicator.

Records of various authors on acid production from sugars, alcohols and allied substances are compared in Table V. Except for D-fructose, glycerol and D-mannitol there were no significant differences in the results. Acid production by *Ps. aeruginosa* from these substances was observed by authors using sensitive methods. Twenty-three of our strains were examined in OF medium and were found to produce acid from these substances. The same strains showed no marked acid reaction in 0.1% peptone water with Andrade indicator.

In defined medium containing inorganic nitrogen sources the breakdown into acid and utilization as carbon source of carbohydrates and allied substances should sharply be distinguished. KLUYVER, DE LEY and RIJVEN [27] showed that some *Pseudomonas* strains produced acid from maltose and lactose but did not grow in defined medium containing these sugars. LIU [36] described that in ammonium salt medium *Ps. aeruginosa* produced acid from glucose, galactose, mannitol, glycerol, arabinose, xylose and mannose, but was able to grow only in tubes containing the first four substances. The difference between acid production and growth was also observed by LYSENKO: while in defined galactose medium 125 out of 126 *Pseudomonas* and related organisms produced acid, only one strain was able to multiply; considerable

differences existed between acid production and growth with 13 further carbohydrates. All these findings have been confirmed in the present study. Acid production in defined medium, however, has not been considered suitable for serving as a standard diagnostic test.

In LYSENKO's opinion [40] the use of growth as an indicator of utilization is difficult as slight growth may occur in the control tubes without carbon source. RHODES [44], who made similar observations, attributed this phenomenon to growth at the expense of intracellular food reserves or of carbonaceous impurities in the medium. JESSEN [21], who also observed a faint turbidity in control media seeded with *Pseudomonas* strains, suggested that the cultures assimilated carbon dioxide adsorbed from the laboratory atmosphere. Other authors using defined media mentioned no similar observations. In the present studies indefinite growth reactions were eliminated by inoculating the media with small amounts of bacteria taken from agar slant cultures which had been left to stand at room temperature for 3 days and by recording positive results only for tubes showing dense turbidity or heavy pellicle formation.

Table VI presents data of authors who considered growth in defined medium as a criterion of utilization. Our own results, except for trehalose utilization, completely agreed with those of STANIER *et al.* [56], although they used a different technique (replica plates incubated for 4 days at 30°C). The difference in trehalose utilization was not significant; the overwhelming majority of our cultures did not grow in trehalose earlier than after 4 days. The results of LIU [36] agree with the above findings except for galactose utilization. In galactose some of our strains developed a late, slightly visible turbidity; this reaction, in comparison to the heavy growth in well-utilizable substrates, was recorded as negative.

The numerous discrepancies between the above data and the findings of RHODES [44] may be explained by technical differences. RHODES sterilized her media by using relatively high temperatures the deleterious effect of which has been pointed out by STANIER *et al.* Other discrepant results, for example those of PULVERER and KORTH, who used Seitz filtration, may perhaps be attributed to differences in the inoculum.

As to organic acids, there is general agreement that *Ps. aeruginosa* utilizes acetate, benzoate, citrate, lactate, malonate and succinate. The opinions disagree for the utilization of formate (positive according to LYSENKO and RHODES, negative according to SELEEN and STARK and the present studies) and also for the utilization of oxalate (positive for some strains according to LYSENKO, uniformly negative in the present studies). The doubtful utilization of these substances was mentioned also by JESSEN. The discrepancies, in addition to differences in the inocula, may have been due to the variable substrate concentrations applied. It is questionable whether it is justified to

define characters on the basis of reactions with such toxic substances, which more or less inhibit the growth of strains at concentrations needed for the clear interpretation of the results.

In view of the above findings, reactions of *Ps. aeruginosa* in sugars, alcohols, organic acids and allied substances may be summarized as follows. Acid production is most suitably tested in media containing low concentrations of complex nitrogenous materials. In these media nearly all strains produced well-demonstrable acid from D-arabinose, L-arabinose, D-galactose, D-glucose, D-mannose and D-xylose. Acid production from L-arabinose, D-galactose, D-glucose and D-xylose could usually be shown even in 1% peptone water. Most strains produced from D-fructose, glycerol, and D-mannitol slight amounts of acid detectable only in sensitive media (defined or OF). A very weak acid production from rhamnose may occur in sensitive media; in view of its difficult reproducibility it has been included in Table V as negative. Part of *Ps. aeruginosa* strains produced well-demonstrable amounts of acid in D-trehalose and L-xylose. Repeated experiments indicated that a considerable part of the cultures gave variably positive and negative reactions (D-trehalose 24.4, L-xylose 24.0%). The use of the two reactions for differentiating biotypes is, therefore, limited. A similar observation was made by JESSEN [21] who concluded that the trehalose reaction is of minor value in the routine diagnosis of *Ps. aeruginosa*.

The proper evaluation of utilization tests is dependent on an adequate technique. JESSEN [21] has come to the conclusion that growth tests are difficult to reproduce and to standardize. If, however, the well agreeing results obtained by different techniques (LIU [36], STANIER *et al.* [56], present work) are considered, an adequate standardization and interpretation of these tests is by no means hopeless. At present it may be stated that acetate, benzoate, citrate, D-fructose, ethanol, glycerol, D-glucose, lactate, malonate, D-mannitol and succinate are readily utilized by almost all *Ps. aeruginosa* strains. Although these utilization tests are unsuitable for an intra-species classification of *Ps. aeruginosa*, some of them — as shown by the extensive studies of STANIER *et al.* using a great number of substrates — may be useful for distinguishing between various species of the genus *Pseudomonas*.

Other reactions associated with carbohydrate metabolism are compared in Table VII. Opinions are uniform that *Ps. aeruginosa* strains fail to hydrolyse starch and are Voges-Proskauer negative. Aesculin is not hydrolysed according to PULVERER and KORTH [42] and the present examinations; LYSENKO [40] described variable results. The methyl red test is positive according to RHODES [44]; however, as she used synthetic medium, her finding is not comparable to other results obtained in the usual medium. Although our cultures have not been examined for gluconate reaction, it may be concluded from the uniform results that all *Ps. aeruginosa* strains are positive.

2. *Nitrogenous substances.* As shown in Table VII uniform results are described for the amino acid tests. Opinions agree also as to proteolytic activity. Apart from some rare exceptions, *Ps. aeruginosa* strains liquefy gelatin. Some authors have noted that freshly isolated strains are more active in this respect. This observation holds true, but by the use of adequate techniques (rapid methods introduced recently) gelatin liquefaction can be demonstrated also in laboratory strains. Casein digestion by *Ps. aeruginosa* has been described as positive and our studies have indicated that Loeffler's serum is also liquefied. There is general agreement that *Ps. aeruginosa* fails to produce indole.

Opinions disagree as to the lecithinase reaction. On egg-yolk medium KLINGE and GRÄF [25], SELENKA [53], LYSENKO [40], JESSEN [21] and STANIER *et al.* [56] obtained negative results for *Ps. aeruginosa* and described the reaction as suitable for the differentiation of this organism from other pseudomonads. In contrast, according to RHODES [45], KLEINMAIER and SCHUBERT [26] and PULVERER and KORTH [42] the egg yolk reaction is positive for *Ps. aeruginosa*. In the present study reactions on the usual egg-yolk medium [5] were not readily interpretable. It has been described that the egg-yolk test depends on various factors and is associated with enzymes of debated nature [49, 66, 67]. There is general agreement as to the positivity of the lipase reaction in *Ps. aeruginosa*, although various substrates were used (RHODES: olive oil and Tween 80; LYSENKO: olive oil; JESSEN and STANIER *et al.*: Tween 80; present studies: castor oil).

It is also generally acknowledged that *Ps. aeruginosa* reduces nitrate to nitrite and beyond. LIU [37] obtained discrepant results in peptone and in defined medium and recommended the latter as a standard test medium for pseudomonads. In contrast, RHODES [44] observed that many of her isolates grew weakly in defined nitrate medium. In the present experiments the usual nitrate broth was satisfactory. Recent observations (JESSEN, STANIER *et al.*) indicate that *Ps. aeruginosa*, unlike many other *Pseudomonas* species, grows well anaerobically in nitrate medium.

According to GABY and FREE [13], KÖHLER [28], LUTZ *et al.* [39] and KLINGE [23] *Ps. aeruginosa* produces no urease. BÜHLMANN *et al.* [3] and VÉRON [61] described variable reactions. These authors tested their strains in media used for the detection of urease activity in *Enterobacteriaceae*. *Ps. aeruginosa* strains in LYSENKO's medium containing only urea and mineral salts gave a positive reaction [40]. In JESSEN's medium of similar composition less defined, frequently negative reactions were observed [21]. For the detection of urease activity in *Ps. aeruginosa*, STEWART's glucose medium with low concentrations of complex nitrogenous substances [59] or the ammonium salt-glucose medium of PULVERER and KORTH [42] would seem more suitable. RHODES [44] showed positive reactions in galactose-urea medium.

Our medium containing urea as a sole source of nitrogen and glucose as a carbon source gave clear-cut reactions. DE TURK [7] has shown that urease in *Ps. aeruginosa* is an adaptive enzyme. It may, therefore, be supposed that in media providing ample sources of nitrogen from other compounds the otherwise positive urease activity may be variable or negative.

3. *Other characters.* Hydrogen sulphide production varies according to the substrate. In the presence of thioglycollate as a sulphur source PULVERER and KORTH [42] showed uniformly positive reactions. In Kligler medium containing thiosulphate HUGH and LEIFSON [20] demonstrated negative results; RHODES [44], in contrast, described *Ps. aeruginosa* as producing H_2S in Kligler agar. JESSEN [21] who observed in ferrophosphate-gelatin medium only one positive and a few delayed reactions, has concluded that *Ps. aeruginosa* is a H_2S negative organism. The discrepancies may be attributed to variations in the sulphur-bearing amino acid content of peptones. In the present studies the standard H_2S agar of RAUSS and VÖRÖS [43] containing thiosulphate, iron and peptone poor in sulphur-bearing amino acids gave uniformly negative results.

Opinions agree as to catalase activity. As seen in Table VII a large number of strains was examined for oxidase activity. The analogous cytochromoxidase test of GABY and HADLEY [15] was also studied by several authors. GABY and FREE [14] considered this reaction more specific for *Ps. aeruginosa* than KOVÁCS's test. According to LUTZ *et al.* [39] and KÖHLER [29] the cytochromoxidase test may be weakly positive or even negative for *Ps. aeruginosa* strains.

As shown in Table VII most strains produce haemolysin and fluorescent pigment. LYSENKO [40] described the loss of fluorescein production in 20–22% of pseudomonads after freeze-drying. Our *Ps. aeruginosa* strains maintained in stab cultures retained this property.

The biochemically highly uniform members of the species *Ps. aeruginosa* differ in pyocyanin production. According to data in the literature the pyocyanin-producing capacity may be lost and pigment production highly depends on the composition of the medium. GABY [11] showed differences in the pyocyanin production by colonial variants within the same strains. The present studies indicate that the loss of pyocyanin production is not too frequent (5.1% after 7–10 years maintenance). LYSENKO described the loss of pyocyanin production in 2 out of 26 strains after freeze-drying. There are no sufficient data as to the stability of pyocyanin production under natural conditions. This property could perhaps be used for the differentiation of biotypes as pyocyanin positive and negative strains occurred approximately evenly distributed in all serological units.

In this work active motility was examined, thus our data cannot be compared with those of authors using flagella staining. The 326 strains exam-

ined by VERDER and EVANS [60] were invariably motile. JESSEN [21] observed 20 non-motile strains among 354 *Ps. aeruginosa* cultures, but remarked that 5 of the former produced motile mutants. Among our strains only one culture, which failed to develop motility after serial U-tube and broth passages, was regarded as non-motile. With earlier cultures of the same strain a fairly good H serum had been produced which agglutinated strains possessing well-developed flagella. Thus, this strain seems to have lost its motility during laboratory maintenance. JESSEN [21] showed by staining that all strains were polarly flagellated. These observations indicate that non-motile *Ps. aeruginosa* strains occur very rarely.

4. *The effect of incubation temperature and of inhibitory substances; growth on selective media.* Table VII shows that opinions are completely uniform as to the growth temperature of *Ps. aeruginosa*. According to the general conception *Ps. aeruginosa* can be simply and more or less reliably distinguished from other pseudomonads by testing growth properties at 42 and 5°C.

The resistance of *Ps. aeruginosa* to brilliant green, in addition to its excellent growth on brilliant green agar, is known from the paper of GOULD and MCLEOD [16]. At the brilliant green concentration applied in the present study *Ps. aeruginosa* strains grew usually better than most *Enterobacteriaceae* organisms. Resistance of *Ps. aeruginosa* to quaternary ammonium bases is well known [34, 38, 64, 65]. At the cetylpyridinium bromide concentration used none of the *Enterobacteriaceae* strains examined were able to multiply. Although in merthiolate resistance the difference was not so sharp between *Ps. aeruginosa* and *Enterobacteriaceae*, in our previous studies merthiolate and brilliant green were successfully combined in a selective enrichment medium for culturing *Ps. aeruginosa* from faecal specimens [34]. According to SELEEN and STARK [51], HUGH and LEIFSON [20] and the present studies, *Ps. aeruginosa* is resistant to potassium cyanide. The tolerance of *Ps. aeruginosa* to high sodium chloride and bile concentrations has long been known. Its resistance to triphenyltetrazolium chloride described by SELENKA [52, 53] has been confirmed by WAHBA and DARREL [63] and the present studies.

There are few data for the growth of *Ps. aeruginosa* on selective and differential media used in routine examinations. This is explained by the fact that in the clinical laboratory *Ps. aeruginosa* strains are generally isolated on simple media. WETMORE and GOCHENOUR [64] recommended selective media for distinguishing between *Ps. aeruginosa* and the genus "Malleomyces". For culturing *Ps. aeruginosa* from faeces and other materials containing a great number of miscellaneous bacteria the use of selective media is necessary. Accordingly, it has been considered advisable to describe the cultural characters of the organism on these media. Colonial morphology on simple agar has not been dealt with, partly because there are many observations in this respect, partly because, in agreement with ZIERDT and SCHMIDT

[68], it has been found that even primary cultures dissociate easily to various colonial forms.

5. *General conclusions.* Data for the biochemical and other characters of *Ps. aeruginosa* obtained by essentially similar methods and being consequently comparable with one another and with the present findings have been summarized in Tables V, VI and VII. As the data reflect examinations of a relatively great number of strains, it seemed justified to define each character in percentage distribution of positive and negative isolates.

Tables V, VI and VII indicate that *Ps. aeruginosa*, this widely distributed species, is highly homogeneous in biochemical and cultural characters. If pyocyanin production, an inconstant property in the opinion of some authors, and reactions in D-trehalose and L-xylose shown to be variable in many strains, are disregarded, the commonly used tests provide no means for distinguishing biotypes within the species. Recently STANIER *et al.* [56] have shown differences between *Ps. aeruginosa* strains in amino acid and other utilization tests not yet used in classification studies. Further investigations may, accordingly, reveal some reactions suitable for the subdivision of the species.

It has been shown that in biochemical and cultural properties there is no difference between the serological units of *Ps. aeruginosa*; the distribution of atypical cultures or strains giving variable reactions with some tests is roughly uniform in all serogroups. In respect to biochemical homogeneity, *Ps. aeruginosa* differs considerably from other Gram negative rods widely distributed in nature.

It may be concluded that although data for the biochemical and cultural characters of *Ps. aeruginosa* are important for classification purposes, a practical intraspecies differentiation of the strains is at present possible only by serological methods. The present studies have not been extended to the examination of other pseudomonads. The results, however, confirm the opinion of those authors who regard *Ps. aeruginosa* as a separate taxonomic unit.

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MAINTENANCE OF TOXOPLASMA GONDII IN PRIMARY HUMAN AMNIOTIC CELL CULTURE

By

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Summary. A simple method has been elaborated for the maintenance of *T. gondii*. In the course of 45 passages in 8 months the organism showed no change in morphology, growth ability and animal pathogenicity. The method is considered suitable for the continuous maintenance of toxoplasma without intermediate animal passages. A method using trypsinization has been elaborated for releasing great amounts of toxoplasma cells from the tissue culture debris.

The maintenance of toxoplasma strains in animals presents considerable economic and technical difficulties. Elaboration of methods allowing a continuous maintenance of the strains *in vitro* as safe as animal inoculation has long been the aim of investigations [1—3]. Continuous cultivation of toxoplasma has become a reality after the introduction of monolayer tissue cultures into microbiological technique. In the last 15 years several authors described the maintenance of toxoplasma in various tissue cultures [4—7]. In a previous paper we showed that toxoplasma strains exhibit a striking affinity to human primary amniotic cell cultures [8]. In the present paper an account is given of the results obtained during long-term maintenance of toxoplasma by this method.

Materials and methods

Toxoplasma strain. *T. gondii* type strain RH was used. Tubes of the first serial culture were inoculated with the peritoneal exudate of mice as described previously [8]. The cell count was determined in a haemocytometer and each tube was inoculated with 1 million organisms.

Tissue culture. Primary human amniotic cell cultures were prepared by the usual trypsinization method. Hanks' solution containing 10% human serum and 0.4% lactalbumin hydrolysate was used as nutrient medium. The medium was changed at 5 to 6 day intervals and before inoculation.

After inoculation the cultures were incubated at 37°C for 3 days then stored at 4°C for 2 days. Subsequently the tissue was scraped off the tube wall with a glass rod. In order to remove toxic metabolic products of toxoplasma and tissue culture the material was centrifuged. The deposit was resuspended in 1 ml fresh nutrient medium and 0.2 ml aliquots were transferred to each tube of the second series and incubated as indicated above. Further passages were performed in the same manner.

To elaborate a method allowing an evaluation of growth ability more reliable than the qualitative technique used in our previous work, the following experiments were performed. Each tube of a series was inoculated with a given number of toxoplasma cells incubated at 37°C for 72 hours, the cell counts were determined, then 0.25 ml of 1% Difco trypsin was added to the supernatant (0.2% final concentration). The trypsinized cultures were incubated

at 37°C and the cell count in the supernatant was determined after 2 and 4 hours. To examine the effect on toxoplasma of trypsin the enzyme was removed by centrifugation of cultures treated for 2 and 4 hours then cell counts were determined and 0.2 ml aliquots of the suspension were inoculated into new tubes. After 72 hours incubation the subcultures were subjected to a similar trypsin treatment and the cell counts were determined after 2 and 4 hours incubation.

Virulence. Pathogenicity of the organism maintained for 8 months in amniotic tissue culture was determined by inoculating mice with 1 ml amounts of serially diluted tissue culture trypsinized for 1 hour. The LD₅₀ was calculated from the number of deaths.

Results

Microscopic examination indicated that in 72-hour tissue cultures 60 to 90% of cells remaining on the tube wall contained large numbers of toxoplasma. Although the infection caused a severe damage in the tissue culture the degree of detachment from the tube wall was slight. In spite of intense

Table I

Number of toxoplasma cells per ml tissue culture before and after trypsin treatment

Tube	Toxoplasma count/ml (log ₁₀)		
	Before trypsinization	After 2 hours trypsinization	After 4 hours trypsinization
1	6.41	7.09	7.17
2	6.50	7.05	7.14
3	6.20	6.51	6.58
4	6.34	6.89	6.90
5	6.25	6.43	6.51
6	6.15	6.46	6.60

multiplication, the toxoplasma count in the supernatant was always low, because most of the organisms remained embedded in the cellular debris on the tube wall. Scraping off the culture yielded a toxoplasma count suitable for further passage. The age of the tissue culture had no influence on the degree of multiplication and in the course of the maintenance of the strain good growth was always obtained. Trypsin treatment of the cultures generally improved the inocula; it has been shown several times that the toxoplasma cell yield was 5 to 8 times higher in trypsinized than in untreated cultures.

Trypsinization is very suitable for releasing toxoplasma organisms from the cellular debris. Before trypsin treatment very few toxoplasma cells were present in the haemocytometer fields while 2 hours after trypsinization a homogeneous, easily countable toxoplasma suspension was obtained. Maximum counts were observed after 4 hours treatment when the cellular debris detached

Table II*Effect of trypsin on the growth ability of toxoplasma*

First passage; inoculum per tube, 5.54

Tube	Toxoplasma count/ml ($\log_{10}$)		
	Before trypsinization	After 2 hours trypsinization	After 4 hours trypsinization
1	5.76	5.99	6.30
2	5.86	6.17	6.23
3	5.65	5.71	5.86
4	5.63	6.20	6.25

Second passage; inoculum per tube, 5.71

Tube	Before trypsinization	After 2 hours trypsinization	After 4 hours trypsinization
1	6.30	6.53	6.63
2	6.17	6.27	6.46
3	6.32	6.47	6.47
4	6.38	6.43	6.47

Table III*Animal pathogenicity of toxoplasma strain RH maintained for 8 months in human amniotic tissue culture*

Experiment	No. of mice	No. of toxoplasma cells inoculated	No. of mice	
			died	survived
1	3	370 000	3	—
	3	37 000	3	—
	3	3 700	2	1
2	12	2 000	12	—
	12	1 000	12	—
	12	500	12	—
	12	100	6	6

perfectly from the tube wall. An example of the numerous experiments performed on the effect of trypsin treatment is presented in Table I.

Subculturing of parasites exposed to 2 or 4-hour trypsin treatment indicated that at the given concentration the enzyme exerted no deleterious effect on the growth ability of the organism (Table II). The intactness of toxo-

plasma cells was also shown by their brisk motility at haemocytometric counting.

The virulence of our toxoplasma strain was high after 8 months passage on amniotic tissue. As shown in Table III the LD₅₀ for mice was 100.

Discussion

Several experiments have been performed to maintain toxoplasma *in vitro*. SABIN and OLITSKY [1] maintained the organism in two passages in ground chick embryos suspended in Tyrode solution. CHERNIN and WELLER [3] made 15 passages in roller-tube cultures of mouse embryo tissue. SCHUHOVA [4, 6] succeeded in maintaining the toxoplasma strain CB for 33 months by chronic infection of HeLa cultures. Other authors described successful results on monkey kidney and Hep-2 cell cultures [5, 8]. Our examinations indicated that toxoplasma strain RH could be maintained in primary human amniotic culture without a decrease in growth ability or in virulence. Despite a long passage in tissue culture, very low cell counts were sufficient for causing fatal infection in mice. The incomplete recovery of the intracellular parasites was a disadvantage of tissue culture maintenance of toxoplasma. SCHUHOVA [4] applied trypsin treatment for HeLa cultures. In our experiments various concentrations of trypsin and various exposure times were examined. The results indicated that an 0.2% concentration of trypsin and incubation for 4 hours yielded optimal results. Trypsin under these conditions deteriorated the cellular debris harbouring the toxoplasma cells but exerted no injurious effect on the microorganism. After the removal of trypsin and lysed cellular material a homogeneous toxoplasma suspension is obtained which is suitable for preparing antigen or for conducting biochemical tests.

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VIRULENCE OF SHIGELLA STRAINS ISOLATED FROM DYSENTERIC PATIENTS AND SYMPTOMLESS CARRIERS

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Summary. *Shigella* strains isolated from patients and carriers were compared for virulence by the guinea pig conjunctival test. There was no quantitative difference in the virulence of strains isolated from different sources. The ID_{50} value for a *Sh. flexneri* 3a strain isolated from a symptomless carrier was higher by one exponent than the average ID_{50} for *Sh. flexneri*. The average ID_{50} was in both groups of persons one exponent higher for *Sh. sonnei* than for *Sh. flexneri*. The experimental results indicated that symptomless carriers are as dangerous potential sources of infection as dysenteric patients.

Symptomless carriers are generally regarded as playing an important role in the transmission of dysentery [1, 2]. There are, however, many observations that among healthy persons there are often shigella carriers without causing infection. It has been described that avirulent shigellae are excreted by all [3] or by part of [4] symptomless carriers. The purpose of the present work was to examine the virulence of shigellae excreted by carriers and to compare in this respect to organisms isolated from typical dysentery.

Materials and methods

Rectal swabs taken from symptomless carriers were streaked onto desoxycholate citrate (DC) agar plates within 1 to 2 hours after sampling. Colonies suspect of shigellae were transferred to Russel medium and the usual serological and biochemical tests were performed. Virulence was examined by SERÉNY's conjunctival test [5]. For simple virulence testing each animal was infected with a loopful culture (10^9 cells). Quantitative virulence determination was carried out by preparing a 3-fold dilution series of a bacterial suspension harvested from Roux flasks. Groups of 5 guinea pigs were used. Both eyes of each animal were inoculated with 3 subsequent dilutions of each strain. The degree of virulence was calculated from the size of the dose and the number of eyes reacting positively. Statistical analysis was performed by the probit test. For comparing the virulence of strains the $\log ID_{50}$ values and the confidence intervals were calculated.

Simple virulence testing was carried out within 4 days after taking the specimen. During this period the organism underwent passages on DC, Russel and nutrient agar media. Quantitative determination required an additional day and passage on Roux flask agar. These conditions were strictly observed in order to prevent — or at least to make uniform — the alteration in the virulence of the cultures. Materials from patients with typical dysentery were supplied by the Central Infectious Disease Hospital, Budapest. Symptomless carriers were detected on screening examination of various communities and individual persons. Most communities were examined after the ceasing of acute infections; two of them were screened when new infections still occurred.

Results

A total of 5380 specimens was examined from 1659 symptomless persons. Shigellae were cultured from 63 persons. In 8 persons 2, in 2 persons 3 and in one person 5 positive samples were recorded. Typical dysentery and enteritis developed in 9 and 6 persons respectively, who had been shown to be symptomless excretors. All strains were virulent by the simple conjunctival test. Seven *Sh. flexneri*, 13 *Sh. sonnei*, 1 *Sh. dysenteriae* 2 and 1 *E. coli* O124 : B17 strain were examined quantitatively and compared with 5 *Sh. flexneri* and 5 *Sh. sonnei* cultures isolated from typical dysentery.

Table I
Virulence of strains isolated from dysenteric patients

Organism	Designation of strain	log ID ₅₀	Confidence interval	
			lower limit	upper limit
<i>Sh. flexneri</i> 1b	64 La	5.59	.	.
<i>Sh. flexneri</i> 1b	200/4 La	5.20	4.34	5.61
<i>Sh. flexneri</i> 1b—3b	310 La	5.39	5.15	5.64
<i>Sh. flexneri</i> 2a	269/6 La	5.79	5.25	5.81
<i>Sh. flexneri</i> 4a	62 La	5.60	4.63	6.03
<i>Sh. sonnei</i>	26 La	6.75	6.40	7.26
<i>Sh. sonnei</i>	63 La	7.01	.	.
<i>Sh. sonnei</i>	288/7 La	6.90	5.33	8.95
<i>Sh. sonnei</i>	867 La	6.72	.	.
<i>Sh. sonnei</i>	993 La	6.70	6.38	7.02

. = not calculated in view of high standard error

Table I shows the virulence of strains isolated from patients. It is evident that *Sh. flexneri* and *Sh. sonnei* strains differ significantly in ID₅₀ values. A similar difference, in that the ID₅₀ for *Sh. sonnei* was higher by one exponent than for *Sh. flexneri*, was demonstrated in the group of symptomless carriers (Table II).

In view of epidemiological differences in the environment the origin of strains isolated from symptomless carriers is described. *Sh. flexneri* 2a strain V 87 was isolated in a nursery school one month after the end of an outbreak of acute infections. The excreter showed no symptoms during the epidemic period. Strain HP 33 was isolated from a carrier treated in the neurology department of a children's hospital. The child yielded two positive cultures a few days before discharge from hospital. Neither the child nor his fellow

Table II
Virulence of strains isolated from symptomless carriers

Organism	Designation of strain	log ID ₅₀	Confidence interval	
			lower limit	upper limit
<i>Sh. flexneri</i> 2a	V 87	5.09	.	.
" 3a	HP 33	5.83	5.62	6.05
" 3a	HP 33 Kc.	5.73	.	.
" 3a	Kö 80	6.63	6.56	6.70
" 3a	44/1	5.36	4.63	6.94
" 3a	50/4	5.09	.	.
" var. Y	55/1	5.60	5.51	5.70
<i>Sh. sonnei</i>	Vör 8a	7.13	6.60	7.53
"	Vör 11	7.32	7.17	7.48
"	Vör 12a	7.36	.	.
"	Vör 21a	6.91	5.51	9.09
"	Vör 37	6.77	.	.
"	Vör 40	6.67	5.79	7.08
"	Vör 50	6.88	6.65	7.12
"	Vör 61	6.71	6.48	6.94
"	Vör 67	6.79	.	.
"	F 39	6.94	6.81	7.32
"	F 42	6.83	.	.
"	G 77	6.99	.	.
"	Ir-D 56	6.90	6.60	7.02

. = not calculated in view of high standard error

patients displayed enteric symptoms and the environment was bacteriologically negative. Strain HP 33 and its substrain HP 33 Kc obtained after conjunctival passage were identical in the degree of virulence. *Sh. flexneri* 3a strain Kö 80 was isolated from a member of a practically closed community, a children's home, where *Sh. dysenteriae* 2 and *Sh. sonnei* frequently occurred during the observation period lasting for a few months. In the beginning of the examinations the child harbouring strain Kö 80 excreted also *Sh. dysenteriae* 2. The two-week period of *Sh. dysenteriae* 2 excretion was accompanied with mild enteritis and subfebrility up to 37.2°C. Later the symptoms and rectoscopic changes disappeared but excretion of *Sh. flexneri* 3 persisted. Weekly screening examinations in the children's home failed to demonstrate the spreading of *Sh. flexneri* 3. Strain Kö 80 was the only *Sh. flexneri* culture showing an ID₅₀ value higher by one exponent than the average; in other

words, this strain was ten times less virulent than other *Sh. flexneri* strains. The log ID₅₀ for the mentioned *Sh. dysenteriae* 2 strain was also high (7.46). In lack of studies the result could not be compared to any other *Sh. dysenteriae* 2 strain.

Three strains were isolated in regional public health stations, thus the time elapsing between their isolation and virulence testing was somewhat longer than that for strains isolated in our laboratory. Yet, in ID₅₀ these

Table III
Average virulence of strains according to examination groups

Group	log ID ₅₀	Confidence limits
<i>Shigella flexneri</i>		
(a) patients	5.5212	5.1706 5.8216
(b) symptomless carriers (except strain Kö 80) .	5.6345	5.0832 5.9652
<i>Shigella sonnei</i>		
(a) patients	6.7836	6.4066 7.1985
(b) symptomless carriers	6.9386	5.5976 7.2620

strains were similar to our cultures. Strains 44/1 and 50/4 were isolated from an aged symptomless carrier. Strain 55/1 was harboured by another symptomless carrier living in an environment free from shigella infection. All three strains split off some avirulent colonies. In the agar plate culture of strain 55/1 virulent Y variant, avirulent Y variant and avirulent *Sh. flexneri* 2a colonies were distinguished.

Sh. sonnei strains were isolated from regional and factory day-time nurseries or schools at various intervals after the occurrence of acute infections. At the time of the examinations all carriers were symptomless. The history of enteric disease was excluded only in children harbouring strains Vör 40, F 39, F 42, G 77 and Ir-D 56. The rest had had mild infections. As to virulence no difference was revealed between the strains.

Table III shows average ID₅₀ values for cultures according to groups of examined persons. Similarly to data obtained in individuals, the average virulence was at the same level for patients and symptomless carriers. There was, however, a one exponent difference in ID₅₀ between *Sh. flexneri* and *Sh. sonnei* regardless whether the cultures had been isolated from patients or symptomless carriers.

In Table IV the examined communities are grouped according to epidemiological features. In the first group which included communities

where no history of enteric infection had been recorded, the incidence of symptomless carriers was 0 to 0.5%. In the second group several cases of dysentery had occurred before the examination; the incidence of symptomless carriers in this group was 0.75 to 5.5%. Group 3 included two communities where shortly before the examinations several persons had suffered from dysentery and one patient in each of them still exhibiting acute symptoms. The incidence of carriers in group 3 was 7 to 13%. In group 4 where severa

Table IV
Summarized data for screening examinations

Group	No. of individuals examined	No. of examinations	Incidence of symptomless carriers, per cent	Organism isolated
Environment free from shigellae (4 communities)	1103	2651	0 -- 0.5	<i>Sh. flex.</i> 3a <i>E. coli</i> 0124
Dysentery infections before the examination period. Symptomless environment (3 communities)	289	633	0.75— 5.5	<i>Sh. flex.</i> 2a
Some dysentery infections still present (2 communities)	119	414	7 — 13	<i>Sh. sonnei</i>
Dysentery infections prevalent (2 communities)	148	1682	16 — 30	<i>Sh. dys.</i> 2 <i>Sh. flex.</i> 1b, 2a, 3a, 6 <i>Sh. sonnei</i>
Total	1659	5380	0 — 30	

acute infections occurred at the time of the examination (3 in one and 11 in the other community) the incidence of symptomless carriers was higher than that of patients (30 and 20%, respectively).

Discussion

The extensive literature on the epidemiological role in dysentery of symptomless carriers and chronic infections has been reviewed by RUDNAI [6] and SERÉNY [7]. In Hungary, the problem was investigated by BÁRÁSZ [8], RUDNAI and MIHÁLYFI [9] and MÁTÉ [10]. Earlier data collected in the National Institute of Public Health showed the incidence of shigella carriers among healthy food handlers to amount to 1 to 2%. Among nursery children a 6.8 [9], among army personnel a 6.4% incidence was noted. According to RUDNAI the estimated incidence of carriers in the environment of dysenteric patients is 10%. The high incidence of carriers arose a doubt as to their epi-

demiological role, as it had been suggested that shigellae harboured by at least part of the carriers belonged in reality to the normal flora of the intestinal tract. It was also questionable whether symptomless carriers excreted avirulent or slightly virulent organisms.

The present data for the incidence of symptomless carriers (0 to 30% according to the epidemiological character of the examined community) approximated those described in the literature. Cultures isolated from carriers did not differ in quantitative virulence from strains responsible for typical dysentery. The only exception, *Sh. flexneri* strain Kő 80 was significantly less virulent than other *Sh. flexneri* cultures. The observation that this organism did not spread to the environment might be ascribed to its decreased virulence. In guinea pig conjunctival infection the distance between the 100% effective dose and the ineffective dose is about one exponent; the same one exponent difference was revealed between strain Kő 80 and the rest of *Sh. flexneri* cultures. Under natural conditions, however, the one exponent decrease in virulence might be compensated by a higher infective dose. In primary DC cultures from both carriers and patients the number of *Shigella* colonies varied between 1 and 100. In connection with this problem another fact experienced in the guinea pig model should be considered: if a fraction of the effective dose is given, the bacteria can often be cultured from the animals' eyes two, three and sometimes even five days after the infection. Shigellae obtained from these "symptomless excreter" guinea pigs show no difference in virulence from the original strain and from cultures reisolated from manifest keratoconjunctivitis. If it is taken into account that strain Kő 80 was isolated from a child in whose environment dysentery infections had been prevalent, the cause for its not being able to cause dysentery in the carrier and environment is probably associated with immunological factors.

In order to approach the problem from another view, the histological examination of two eyes yielding positive cultures without symptoms was performed. One of the eyes was examined during "carriership" characterized by a decreasing number of excreted bacteria, the other one day after the ceasing of the excretion. Histological examination showed in the cornea and in the conjunctiva (especially at the conjunctival fold) several surface erosions and small intra-epithelial abscesses. In the lumen of the abscesses there were polymorphonuclear leucocytes. In the cytoplasm of epithelial cells at the site of the erosions and of epithelial cells surrounding the abscesses thionine-staining rod-shaped bacteria were present. Symptomless excretion in humans may perhaps be due to similar microlesions.

Our findings indicate that the decreased virulence of shigellae plays no important part in the aetiology of symptomless excretion. Persons harbouring these bacteria without clinical manifestation are as dangerous sources of infection as dysenteric patients.

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REGULATION OF ALKALINE PHOSPHATASE SYNTHESIS IN AUXOTROPHIC MUTANTS OF *BACILLUS SUBTILIS*

By

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Summary. Alkaline phosphatase production in *B. subtilis* was repressed by phosphate concentrations ranging from 0.46 to 0.66 mM. Adenine dependent strains were an exception as even at minimal phosphate ion concentration they exerted a phosphatase activity as low as wild-type strains do after total repression.

Transformation experiments have indicated that the decreased phosphatase activity is due to a mutation in structural genes responsible for adenine biosynthesis. Strains undergoing adenylosuccino synthase and adenylosuccino lyase mutations showed a similar decrease in phosphatase activity.

Adenylosuccino synthase structural gene mutation causing the loss of adenine biosynthesis but not influencing alkaline phosphatase activity was also observed.

Alkaline phosphatase production in *Bacillus subtilis* can be repressed by incorporating inorganic orthophosphate in the medium [1]. Phosphatase synthesis in wild-type strains is regulated by two regulator genes. Injury of either of these genes causes a change in the regulation of the enzyme synthesis manifesting itself in a repressibility different from that observed in the wild-type organism [2].

The damage of adenine and guanine biosynthesis in *B. anthracis* mutants was associated with an altered repressibility of alkaline phosphatase [3]. Similar observations were made in examining purine auxotrophs of *B. subtilis* as adenine dependent strains showed an alteration in phosphatase regulation. Transformation experiments indicated that this finding was attributable to changes in the structural genes of adenine synthesis. The present paper gives an account of these observations.

Materials and methods

Organisms. Bacterial strains of our collection were used. The list of strains presented in Table I was compiled as recommended by DEMEREC *et al.* [4], except that designation *pur* was used only for strains in which the mutation had affected the structural genes of enzymatic processes taking place before the closing of the purine ring. Strains in which the block was between inosylic and adenylic acid were termed as in our previous paper [5], *ade* mutants. Strains showing a damage in adenylosuccino synthase (EC 6.3.4.4.) were designated as *ade I*, those with a damage in adenylosuccino lyase (EC 4.3.2.2.) as *ade II*. The strains were maintained as spore suspensions.

Table I
List of the reference strains of *B. subtilis*

Strain	Source	Genotype	Phenotype (requirement)	Former designation
Sz 1	a	Prototrophic	—	Marburgy Yale
Sz 2	b(Sz 1)	<i>str-1</i>	streptomycin resistant	—
Sz 3	a	Prototrophic	—	W ₂₃
Sz 4	b(Sz 3)	<i>str-2</i>	streptomycin resistant	—
Sz 5	c	<i>adeI-1</i>	adenine	MB 1362
Sz 6	c	<i>adeII-1, phe</i>	adenine, phenylalanine	MB 1364
Sz 7	c	<i>adeI-2, trp</i>	adenine, tryptophan	MB 1473
Sz 8	a	<i>trp</i>	tryptophan	168 ind ⁻
Sz 9	d	<i>trp, his</i>	tryptophan, histidine	168 ind ⁻ his ₃₁ ⁻
Sz 10	e	<i>trp, pur-1</i>	tryptophan, any purine	168 ind ⁻ ad ₁₈ ⁻
Sz 11	e	<i>trp, pur-2</i>	tryptophan, any purine	168 ind ⁻ ad ₂₁ ⁻
Sz 12	e	<i>trp, pyr-1</i>	tryptophan, uracil	168 ind ⁻ ura ₆ ⁻
Sz 13	e	<i>trp, pyr-2</i>	tryptophan, uracil	168 ind ⁻ ura ₂₃ ⁻
Sz 14	f	<i>trp, pyr-3</i>	tryptophan, uracil	168 ind ⁻ ura ₂₆ ⁻
Sz 15	e	<i>thr</i>	threonine	115 thr ⁻
Sz 16	f	<i>trp, leu</i>	tryptophan, leucine	168 ind ⁻ leu ₈ ⁻
Sz 17	f	<i>trp, met</i>	tryptophan, methionine	168 ind ⁻ met ₂₂ ⁻
Sz 18	f	<i>trp, arg</i>	tryptophan, arginine	168 ind ⁻ arg ₂₆ ⁻
Sz 19	f	<i>trp, nic</i>	tryptophan, nicotinic acid	168 ind ⁻ nic ⁻

Source of the strains:

- a = P. SCHAEFFER, Institut Pasteur, Paris.
- b = This laboratory; parent strains in parentheses.
- c = A. L. DEMAÏN, Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey.
- d = S. ZAMENHOF, University of California, Los Angeles.
- e = From the collection of this laboratory; the previous source is unknown.
- f = Isolated from Sz 8 in this laboratory by Dr. E. MARJAI and Dr. T. BAKANCSIKOVA.

Culture media. *LA medium*: Lactalbumin hydrolysate, 0.2 g; MgSO₄, 0.2 g; Tris hydroxyl methyl aminomethane, 4 g; KCl, 2 g; distilled water, 1 litre; the pH was adjusted with N HCl to 7.4. After sterilization the medium was supplemented with glucose (2 mg/ml) and the required amino acids (20 µg/ml) or purine or pyrimidine bases (10 µg/ml). The phosphate concentration in the medium was 0.06 mM. *YP medium*: Complete medium containing yeast extract and peptone [6].

Cultivation methods. For the examination of alkaline phosphatase activity 0.02 M K₂HPO₄ was added to a series of media to contain 0.26, 0.46, 0.66, 0.86, 1.06 and 1.26 mM phosphate. The media were distributed at 10 ml portions into 100 ml Erlenmeyer flasks and inoculated with spores. The flasks were incubated at 37°C for 16 hours on a gyratory shaker operating at 240 r.p.m.

Determination of alkaline phosphatase. The cultures were centrifuged, washed with saline and resuspended in saline to a density of 0.3 mg dry bacteria per ml. To 2 ml suspension

2 ml reagent (0.5 per cent paranitrophenyl phosphate dissolved in 0.2 M pH 8.5 Tris/HCl buffer) were added. After incubation in water bath at 37°C for 10 minutes the reaction was terminated with 1 ml N NaOH, the suspension was centrifuged and the optical density of the supernatant was measured in a Spectromom photometer at 410 m μ . Enzyme activity was expressed as μ M paranitrophenol per ml.

Determination of adenine pathway enzymes. Adenylosuccino synthase and adenylosuccino lyase were determined as described previously [5].

Preparation of DNA. The bacteria were cultured by shaking in 1 litre Erlenmeyer flasks containing 200 ml YP medium. Late exponential phase cultures were centrifuged and DNA was extracted by MARMUR's method [7] as modified in our laboratory [8]. DNA concentration was determined by BURTON's technique [9].

Transformation. The method described by ANAGNOSTOPOULOS and SPIZIZEN was used [10].

Results

Alkaline phosphatase repression. Alkaline phosphatase activity in wild-type *B. subtilis* strains varied according to the phosphate ion content in LA medium (Fig. 1). Bacteria cultured in the presence of 0.26 to 0.46 mM phosphate showed identical enzyme activity. In the range of 0.46 to 0.66 mM phosphate the phosphatase activity decreased. A further increase of the phosphate content resulted in no further change.

The repression curves for streptomycin resistant and amino acid, pyrimidine or nicotinic acid dependent mutants were similar to those for the wild-type organism.

The purine auxotrophs were less uniform in respect to alkaline phosphatase repression. Strains in which the mutation had affected enzymatic steps taking place before the closing of the purine ring (*pur* strains) were similar in phosphatase repressibility to the wild-type strain. Strains *ade* I-2 and *ade* II-1 exhibited repression curves significantly different from those of the above cultures (Fig. 1). Even at the lowest phosphate concentration the phosphatase activity in strains *ade* I-2 and *ade* II-1 was as low as in the wild-type organism after total repression. Increasing the phosphate content in the medium exerted no effect on the low enzyme activity in these mutants.

Strain *ade* I-1 differed from the other *ade* strains in alkaline phosphatase repression and was similar to the wild-type organism.

Phosphatase activity in our strains in the presence of 0.26 and 1.26 mM phosphate is presented in Table II.

Transformation experiments with adenine dependent mutants. Adenine dependent strains showing altered phosphatase repression curves were used as recipient, wild-type strains and adenine auxotrophs as donor organisms. DNA was diluted to produce only 1 to 5% of the number of mutants expected under the experimental conditions (0.05 μ g/ml). Ten adenine independent transformants were selected from each culture and examined for alkaline phosphatase repression. The results are shown in Table III.

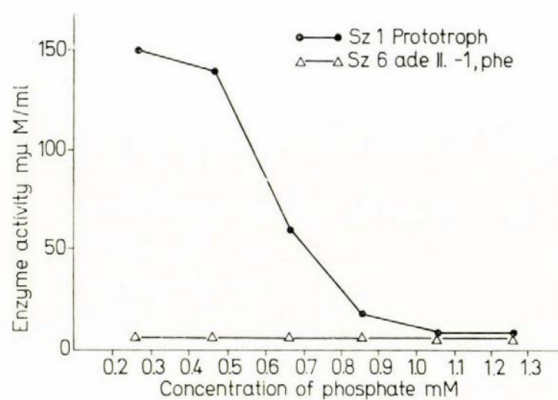


Fig. 1. Repression of alkaline phosphatase synthesis in *B. subtilis*

Table II

Repression of alkaline phosphatase synthesis in *B. subtilis*

Strain	Genotype	Alkaline phosphatase activity in the presence of the media of	
		0.26 mM phosphate	1.26 mM phosphate
Sz 1	Prototrophic	116.3	18.2
Sz 2	<i>str-1</i>	123.2	16.7
Sz 3	Prototrophic	117.4	23.1
Sz 4	<i>str-2</i>	105.2	11.3
Sz 5	<i>ade I-1</i>	112.1	15.4
Sz 6	<i>ade II-1, phe</i>	8.3	4.5
Sz 7	<i>ade I-2, trp</i>	7.1	6.9
Sz 8	<i>trp</i>	127.8	17.6
Sz 9	<i>trp, his</i>	134.2	19.5
Sz 10	<i>trp, pur-1</i>	121.0	21.9
Sz 11	<i>trp, pur-2</i>	113.7	20.4
Sz 12	<i>trp, pyr-1</i>	118.6	14.3
Sz 13	<i>trp, pyr-2</i>	98.2	15.4
Sz 14	<i>trp, pyr-3</i>	107.4	24.7
Sz 15	<i>thr</i>	108.8	10.4
Sz 16	<i>trp, leu</i>	122.2	16.6
Sz 17	<i>trp, met</i>	137.9	17.8
Sz 18	<i>trp, arg</i>	109.1	24.5
Sz 19	<i>trp, nic</i>	128.5	12.6

Table III

Alkaline phosphatase activity in adenine independent transformants

Recipient	Donor (0.05 μ g/ml DNA)	No. of transformants/ 10^8 recipient cells	Average enzyme activity (10 strains) in the presence of the media of	
			0.26 mM phosphate	1.26 mM phosphate
Sz 6	Sz 1	1.2×10^3	108.3	16.2
Sz 7	Sz 1	3.2×10^2	112.3	9.7
Sz 6	Sz 5	4.6×10^2	117.6	13.1
Sz 7	Sz 5	9.0×10	109.4	14.6
Sz 6	Sz 7	1.2×10^2	113.6	18.2

The repressibility of alkaline phosphatase activity was similar in wild-type strains and their adenine independent transformants regardless whether DNA had been prepared from prototrophic or adenine dependent strains. Cross-transformation between adenine auxotrophs characterized by altered repressibility also resulted in transformants similar to the wild-type culture in phosphatase repression.

Discussion

Among auxotrophic mutants of *B. anthracis* those requiring only adenine or guanine and incapable of utilizing other purine compounds showed an altered phosphate regulation [3]. The present investigations revealed a change in phosphatase regulation also in adenine dependent mutants of *B. subtilis*.

The regulation of alkaline phosphatase synthesis was studied by MIKI *et al.* [2]. The structure of the enzyme molecule is determined by a structural gene the function of which depends on two regulatory genes (RI and RII) located at two distant positions on the DNA molecule. Gene RI is linked directly to the structural gene of phosphatase, gene RII is situated farther on the chromosome. Mutation either in the phosphatase structural gene or in the regulatory genes may result in the production of alkaline phosphatase negative mutants.

Two out of our 3 adenine dependent strains showed minimum alkaline phosphatase activity even at the lowest environmental phosphate concentration needed for growth. Enzyme activity in these cultures at minimum phosphate ion concentration was lower than that in wild-type strains after total repression. In phenotype these strains corresponded to alkaline phosphatase negative mutants.

In transformation experiments with these strains alkaline phosphatase-non-producing mutants were used as recipients and prototrophs or adenine auxotrophs exerting normal alkaline phosphatase activity as donors. Cross transformation was performed with the two adenine dependent strains characterized by minimum phosphatase activity. Alkaline phosphatase activity in adenine independent transformants isolated in these experiments was similar to that shown in wild-type strains (Table III).

From findings indicating that the loss of adenine dependency is always accompanied by the normalization of phosphatase activity it may be concluded that the same genetic site is responsible for adenine requirement and altered phosphatase activity. Sites influencing phosphatase activity are present in the structural genes of both adenylosuccino synthase (Sz 7, *ade* I-2, *trp*) and adenylosuccino lyase (Sz 6, *ade* II-1, *phe*).

The damage of these structural genes does not always cause a change in phosphatase synthesis: for example, strain Sz 5 *ade* I-1 was similar to wild-type strains in phosphatase production. Purine auxotrophs of *B. anthracis* also exhibit an altered phosphatase activity but some of them resemble wild-type strains in this respect [3].

These findings present a further example of the observation that the damage in one structural gene may result in a double effect, namely, in addition to a change in its own enzyme protein an alteration occurs also in the function of a different enzyme system. In recent years such changes have been described for *E. coli* arabinose [11, 12] and galactose systems [13], for the sporulation of *B. subtilis* [14], and for *B. subtilis* alkaline phosphatase synthesis [15].

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SIGNIFICATION OF LYMPHOPENIA IN THE PATHOGENESIS OF WASTING SYNDROME IN NEONATALLY THYMECTOMIZED MICE

H. SZERI, P. ANDERLIK, S. BÁNOS

ЗНАЧЕНИЕ ЛИМФОПЕНИИ В ПАТОГЕНЕЗЕ „WASTING SYNDROM“-А, СОПУТСТВУЮЩЕГО ТИМЭКТОМИИ У НОВОРОЖДЕННЫХ МЫШЕЙ

Х. СЕРИ, П. АНДЕРЛИК, Ш. БАНОШ

В новорожденном периоде мыши с удаленной вилочковой железой на воздействие холодом реагируют иначе, чем нормальные животные; они повышено чувствительны к холоду и после стресса дают лимфопению пониженных размеров. Можно предполагать, что вилочковая железа, кроме иммунологической адаптации, играет первостепенную роль и в общих адаптационных процессах.

EFFECT OF HYPERBARIC OXYGENATION ON THE LOCAL SHWARTZMAN PHENOMENON

T. SZILÁGYI, S. TÓTH, L. MILTÉNYI, G. JÓNA

ВЛИЯНИЕ КИСЛОРОДА ПОД ВЫСОКИМ ДАВЛЕНИЕМ НА МЕСТНЫЙ ФЕНОМЕН ШВАРЦМАНА

Т. СИЛАДЫ, Ш. ТОТ, Л. МИЛТЕНЬИ, Г. ЙОНА

Феномен Шварцмана на кроликах, содержащихся в чистом кислороде под давлением в 3 атм, развивался намного быстрее и был больших размеров, чем в контрольной группе, которая содержалась в воздухе под давлением в 1 атм. Гипероксия сама по себе не вызывала феномена Шварцмана, но энергично повышала действие дозы выделяющегося эндотоксина. Оксигенизация при высоком давлении не оказывают влияния на реакцию Томаса-а (адреналин + эндотоксин). Авторы считают вероятным, что гипероксия, повреждая лизозомную мембрану, усиливает развитие и интенсивность реакции Шварцмана.

CULTIVATION OF *TOXOPLASMA GONDII* IN PRIMARY HUMAN AMNION CELL CULTURE

R. CSÓKA, G. KULCSÁR

КУЛЬТИВИРОВАНИЕ *TOXOPLASMA GONDII* НА ПЕРВИЧНОЙ КЛЕТОЧНОЙ КУЛЬТУРЕ АМНИОНА ЧЕЛОВЕКА

Р. ЧОКА, Г. КУЛЧАР

Изучалась чувствительность первичных клеточных культур амниона человека к *Toxoplasma gondii*. Чувствительность культуры сравнивали с чувствительностью культуры HeLa, наиболее часто применяемой в исследовании токсоплазмы. Для оценки степени размножения в случае обоих видов культур подсчитывалось количество внутриклеточных токсоплазм. Было найдено, что для размножения токсоплазм первичная культура амниона человека гораздо более пригодна, чем клеточная культура HeLa.

STUDIES ON SUCROSE BREAKDOWN BY INVERTASE-PRODUCING CLAVICEPS STRAINS

T. PERÉNYI, E. UDVARDY-NAGY, E. K. NOVÁK

ИЗУЧЕНИЕ РАЗЛОЖЕНИЯ САХАРОЗЫ ШТАММАМИ CLAVICEPS, ПРОДУЦИРУЮЩИМИ ИНВЕРТАЗУ

Т. ПЕРЕНЬИ, Е. УДВАРДИ-НАДЬ, Е. К. НОВАК

В отношении трех штаммов *Claviceps*, продуцирующих инвертазу, выяснилось, что их β -D-фруктофуранозидазы способны трансферировать фруктозилные части и таким способом в период разложения сахарозы синтезировать олигосахариды. Расхождения между тремя штаммами выявились по числу трансфер-олигосахаридов, по их количеству и порядку образования. Соответственно предварительным исследованиям, проведенным до сих пор, во всех четырех продуктах преобладает фруктоза, и два из них обладают редуцирующей способностью.

Была дана оценка значению трансфер-олигосахаридов, образующихся в период разложения сахарозы, с точки зрения обмена веществ интактного мицелия штаммов.

PRODUCTION OF CLAVINE-ALKALOIDS BY CLAVICEPS FUSIFORMIS (LOVELESS) IN SUBMERGED CULTURES

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ОБРАЗОВАНИЕ ШТАММАМИ CLAVICEPS FUSIFORMIS (LOVELESS) КЛАВИН-АЛКАЛОИДОВ В ПОГРУЖЕННОЙ КУЛЬТУРЕ

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Штаммы *Claviceps paspali* и *Claviceps purpurea* только тогда продуцируют в погруженной культуре алкалоиды, если культура имеет склероциальную морфологию. В отличие от этого штамм *Claviceps fusiformis* (Loveless) продуцирует алкалоиды только наряду с образованием конидий. Это последнее наблюдение не отрицает, а даже делает более общей взаимосвязь между склероциальной морфологией и образованием алкалоидов, так как — соответственно настоящим исследованиям — естественный склероций *Claviceps fusiformis* содержит плодородное тело и внутри него — конидии.

EFFECT OF CYTOSTATICS ON THE REPLICATION OF VACCINIA, PSEUDO-RABIES AND SINDBIS VIRUSES AND ON THE INTERFERON PRODUCTION BY TISSUE CULTURE CELLS

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ДЕЙСТВИЕ ЦИТОСТАТИЧЕСКИХ СРЕДСТВ НА РАЗМНОЖЕНИЕ ВИРУСОВ ВАКЦИНИИ, ПСЕВДО-БЕШЕНСТВА И SINDBIS, А ТАКЖЕ НА ПРОДУКЦИЮ ИНТЕРФЕРОНА КЛЕТКАМИ ТКАНЕВОЙ КУЛЬТУРЫ

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В клеточной культуре фибробластов куриного эмбриона было исследовано действие многих цитостатических средств на размножение вирусов вакцинии, псевдо-бешенства и Sindbis, а также на образование интерферона. Размножение вирусов вакцинии и псевдо-бешенства подавлялось в оцениваемой степени Degranol-ом (манномустин), Endoxan-ом (цикло-фосфамид), Mannitmyleran-ом (маннитил димезилат), 6-mercaptapurin-ом, Mitomen-ом (хлорметин), Sanamycin-ом (кактиномицин) и Teropterin-ом (теропртерин), а размножение вируса Sindbis — только Degranol-ом (манномустин). На основе изложенного действия средств на продукцию интерферона авторы установили, что подавление последней может быть успешно применено в качестве пробы на токсичность *in vitro* в опытах по вирусной химиотерапии.

SIGNIFICANCE OF STAPHYLOCOCCAL FATTY ACIDS IN THE PATHOGENESIS OF INFLAMMATION

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РОЛЬ ЖИРНЫХ КИСЛОТ STAPHYLOCOCCUS AUREUS В ПАТОГЕНЕЗЕ ВОСПАЛЕНИЯ

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Авторы изучали изменения в составе липоидов почечной и легочной ткани мышей зараженных *Staphylococcus aureus*, спустя различное время после заражения. Было установлено:

1. После заражения количество свободных жирных кислот в воспаленной почке нарастает. Это нарастание пропорционально тяжести воспаления и в 3—6 раз выше по сравнению с контролем.
2. Главным компонентом свободных жирных кислот являются масляная и *cis-vaccen* кислоты, нарастание которых удваивается после заражения компактным вариантом штамма „Smith” *Staphylococcus aureus*, а в течение более тяжелого заражения диффузным вариантом их количество увеличивается в 8 раз.
3. Подобным образом можно продемонстрировать нарастание свободных жирных кислот в воспаленной легочной ткани мышей, зараженных внутриплеврально *Staphylococcus aureus*. Содержание ненасыщенных свободных жирных кислот в воспаленной легочной ткани возрастает в 3 раза по сравнению с контрольной тканью легких.
4. Авторы указывают на значение умноженных жирных кислот в патогенезе воспаления.

VIROLOGICAL EXAMINATION OF CHILDREN WITH ACUTE EXANTHEMATOUS DISEASE

М. Тóтн

ВИРУСОЛОГИЧЕСКОЕ ОБСЛЕДОВАНИЕ БОЛЬНЫХ С ОСТРЫМИ ЗАБОЛЕВАНИЯМИ, СОПРОВОЖДАЮЩИМИСЯ СЫПЬЮ

М. ТОТ

Для выяснения этиологии было проведено бактериологическое и вирусологическое обследование 236 больных со свежей экзантемой, принятых отделениями-изоляторами больницы Ласло за период с октября 1965 года по ноябрь 1966 года. Выделение вируса проводилось из глоточных смывов и стула на первичной клеточной культуре почки обезьяны, на перманентной III/1 культуре почки обезьяны, на первичной культуре почки эмбриона человека, на клеточных культурах RK-13 и HeLa. Предоставилось возможным исследовать парные сыворотки от 159 больных, в случае которых — в зависимости от количества сыворотки — определялись антистрептолизин-титр, титр в РПГА с коревым антигеном, титр в реакции связывания комплемента с аденовирусным антигеном и нейтритализационный титр против вируса краснухи; далее, определялись нейтритализационные титры сывороток больных, выделяющих вирус, против гомологичного вируса.

Весь материал, связанный с экзантемой, принимая во внимание клиническую картину заболевания, был распределен следующим образом: скарлатина 88 (37,3%), корь 63 (26,7%), ветряная оспа 46 (19,5%), атипичная экзантема 39 (16,5%).

В «скарлатинной» группе инфекция, вызванная *Streptococcus haemolyticus*, была подтверждена в 23,8%, 6 случаев оказались корью, а 6 — краснухой. Далее, в 7 случаях причиной инфекции явились аденовирусы, в 10 случаях — энтеровирусы и в 1 случае — вирус парагриппа 3 типа. Среди больных с кореподобной экзантемой в 30% случаев была установлена корь. В 44,7% случаев на основе отрицательной РПГА диагноз кори был исключен. Из них выделение вируса и сероконверсию удалось установить: в 2 случаях — краснуха, в 5 случаях — аденовирус, в 14 случаях — энтеровирус.

Из глоточных смывов больных ветряной оспой удалось выделить на перманентной III/1 клеточной культуре почки обезьяны 12 штаммов *Herpesvirus varicellae*, из которых 10 могли быть выделены и на культуре RK-13. Методом выделения вируса и серологическим путем диагноз ветряной оспы был подтвержден в 45,7%.

Среди 39 случаев атипичной экзантемы 3 оказались краснухой, а 1 — корью. Далее, в 4 случаях инфекция была вызвана вирусом Коксаки, в 3 — аденовирусом, в 2 — вирусом *Herpes simplex* и в 1 случае — вирус RS. В 7,6% имело место свежее заражение *Streptococcus haemolyticus*.

В качестве контроля экзантемной группы в тот же период времени и подобными методами были обследованы 222 неэкзантемных больных подобного возраста.

Было предположено, что существует этиологическая связь между выделением энтеровируса + сероконверсия и экзантемной клинической картиной; о первичном или вторичном наличии аденовирусных инфекций, однако, не могли судить.

TAXONOMIC STUDIES ON MYCOBACTERIA ON THE BASIS OF THEIR ANTIGENIC STRUCTURE

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ТАКСОНОМИЧЕСКОЕ ИЗУЧЕНИЕ МИКОБАКТЕРИЙ НА ОСНОВЕ ИХ АНТИГЕННОЙ СТРУКТУРЫ

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1. Авторы изучали антигенную структуру 8 видов микобактерий иммунной диффузией в гомологичной и гетерологичной системах. На основе выявленных общих антигенных компонентов исследовали родство между штаммами. Изучаемые виды были подразделены на две группы. В группу А относились *M. bovis* BCG, *M. kansasii*, *M. avium*, *M. balnei*, *M. simiae*, которые содержали общий антигенный компонент, минимально

характерный для этой группы, *M. phlei*, *M. smegmatis* на основе общего специфического антигена входили в группу Б. *M. fortuitum* не принадлежит ни к одной из этих групп, хотя близко стоит к *M. smegmatis*.

2. На основе своей антигенной структуры *M. simiae* № 61 п. sp. стоит более близко к *M. kansasii* и *balnei*, но имеет больше антигенов, общих с сапрофитными штаммами (*M. phlei*, *M. smegmatis*), чем другие микобактерии, относящиеся в группу А.

3. Из специфических иммунных сывороток путем истощения гетерологичными антигенами производили так называемые моноспецифические сыворотки, которые практически дают преципитацию только с антигеном, характерным для гомологичного вида. Применение абсорбированных сывороток делает возможным идентификацию некоторых неизвестных штаммов.

SATURATED FATTY ACIDS IN POLIOVIRUS HOST CELL INTERACTION I. STIMULATION AND INHIBITION OF VIRION UPTAKE

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РОЛЬ НАСЫЩЕННЫХ ЖИРНЫХ КИСЛОТ ВО ВЗАИМОДЕЙСТВИИ ВИРУС ПОЛИОМИЕЛИТА - КЛЕТКА

I. Ускорение и торможение проникновения вириона

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Авторы изучали насыщенные парные жирные кислоты с 12—24 углеродными атомами с точки зрения их влияния на проникновение в клетку вируса полиомиелита, связанного с клеткой. В опытах использовался постоянный клеточный штамм почки обезьяны. Действие 3×10^{-3} М бовинальбумина принимали за 100, эффект остальных сравнивался с этим.

Кривые доз действия различных кислот показывают, что при длине углеродной цепочки до 18 атомов эффект не превышает 50% при любой концентрации. Жирная кислота с 24 углеродными атомами (лигноцериновая кислота) в изученных зонах концентрации не оказывала никакого действия. Эффект от *arachin* (C 20)- и *bechen* (C 22)-кислот был приблизительно около 90% при концентрациях 10^{-5} и 10^{-7} мол. Кривые доз действия обеих жирных кислот имели форму колокола; это указывает на то, что с изменением концентрации ускоряющее действие может переходить в подавляющее.

В присутствии любой изучаемой жирной кислоты после 3-х-часовой инкубации при 37° С инфективность вирионной суспензии не изменялась.

После часовой инкубации при 37° С в присутствии любой жирной кислоты клетки не были способны адсорбировать вирион из окружения, свободного от жирных кислот. Подавляющее действие понижалось, если клетки продолжительное время (более 30 мин.) инкубировались в условиях отсутствия жирных кислот.

Была сделана попытка дать возможное объяснение действию жирных кислот.

SATURATED FATTY ACIDS IN POLIOVIRUS HOST CELL INTERACTION II. MODEL EXPERIMENT ON STIMULATION OF PHAGOCYTOSIS

Z. NAGY and A. KOCH

РОЛЬ НАСЫЩЕННЫХ ЖИРНЫХ КИСЛОТ ВО ВЗАИМОДЕЙСТВИИ ВИРУС ПОЛИОМИЕЛИТА - КЛЕТКА

II. Модель-опыт по ускорению фагоцитоза

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Клетки перманентного штамма клеточной почки обезьяны (ПМК III/1) при стимулировании их 3×10^{-5} мол. V фракцией *bovinalbumin*-а или кислотами, арахидиновой или пальмитиновой в концентрации 10^{-9} — 10^{-5} мол., «пожирали» частицы *dowex* в значитель-

ном количестве. Нестимулированные клетки только слабо «заглатывали» частицы *dowex*. Обработка жирной кислотой делала цитоплазму более светлой и гомогенной. *Bovinalbumin* вызывал образование больших вакуолей в крайней части клетки, на периферии однослойной клеточной мембраны.

Ускорение присоединения частиц *dowex*, казалось, стояло вне связи от концентрации изучаемых материалов внутри зоны 10^{-5} — 10^{-9} и не зависело от времени дачи. В этом механизм действия материалов, связанный с ускорением фагоцитоза, представляется отличным от действия, ускоряющего заражение в системе вирус полиомиелита — клетка.

STREPTOMYCIN-LIKE COMPOUND ENZYMICALLY RELEASED FROM A STREPTOMYCIN-NON-PRODUCING STREPTOMYCES GRISEUS STRAIN

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СТРЕПТОМИЦИНОПОДОБНОЕ ВЕЩЕСТВО, ВЫДЕЛЕННОЕ ЭНЗИМАТИЧЕСКИМ ПУТЕМ ИЗ ШТАММА STREPTOMYCES GRISEUS, НЕ ПРОИЗВОДЯЩЕГО СТРЕПТОМИЦИН

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Из мицелия штамма *Streptomyces griseus*, не вырабатывающего стрептомицин, с помощью лизозима можно было выделить вещество, располагающее антибиотической активностью. Идентификация вещества была проведена хроматографией на бумаге. При отождествлении использовался стрептомицин-зависимый штамм *Bacillus megatherium*, а также резистентные и чувствительные к стрептомицину штаммы *Bacillus subtilis*. По данным исследований выделенное вещество подобно стрептомицину.

При гидролизе с пикриновой кислотой мицелия *Streptomyces griseus*, не производящего стрептомицин, также можно было выделить вещество, располагающее антибиотической активностью.

Изучалась зависимость между содержанием углеводорода в изолированной клеточной стенке штаммов *Streptomyces griseus* и способностью штамма вырабатывать стрептомицин.

INDUCTION AND MULTIPLICATION OF λ -PHAGE. II. THE EFFECT OF VALINE AND ISOLEUCINE

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ИНДУКЦИЯ И РАЗМНОЖЕНИЕ λ -ФАГА. II. ДЕЙСТВИЕ ВАЛИНА И ЛЕЙЦИНА

И. ГАДО, Г. САВЧЕНКО, И. ХОРВАТ

После индукции митомицином производство фага в состоянии „shift down” осуществляется до конца, до того как в неиндуцированной культуре начнется размножение клеток. Валин и лейцин в концентрации, подавляющей клеточное размножение, тормозят производство фага. Если это тормозящее действие аминокислот устраним добавлением казеин-гидролизата, lag-фаза при производстве фага в значительной степени сократится по сравнению с индукцией, проведенной одновременно с добавлением казеин-гидролизата. Это указывает на то, что во время торможения происходит несколько стадий размножения фага.

FIXATION AND SENSITIZING CAPACITY IN THE GUINEA PIG OF HORSE IgA, RABBIT AND CHICKEN IgG

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ИССЛЕДОВАНИЕ НА МОРСКИХ СВИНКАХ ТКАНЕВОЙ ФИКСАЦИИ И СЕНСИБИЛИЗИРУЮЩЕЙ СПОСОБНОСТИ ЛОШАДИНОГО IgA, КРОЛИЧЬЕГО И ЦЫПЛЯЧЬЕГО IgG,

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Кроличий IgG, лошадиный IgA и цыплячий IgG в одинаковой степени фиксируются на различных тканях морской свинки. Кроличий IgG из исследованных органов в наибольшем количестве фиксировался на легких. Если повышались дозы лошадиного IgA и цыплячьего IgG, то во всех исследованных органах повышался уровень связанных антител. Хотя легкие морской свинки на действие повышенной дозы антител способны фиксировать такое же количество лошадиного IgA и цыплячьего IgG, как и кроличьего IgG, эти два вида антител всё-таки не сенсibiliзируют животных. Смертельный шок развивается только после инъекции кроличьего IgG на введение гомологичного антигена. Всё это указывает на то, что сенсibiliзирующая способность антител не зависит от степени фиксации на тканях.

INCIDENCE OF RUBELLA VIRUS NEUTRALIZING ANTIBODY IN DIFFERENT AGE GROUPS

M. Tóth

ЧАСТОТА ВСТРЕЧАЕМОСТИ АНТИТЕЛ, НЕЙТРАЛИЗУЮЩИХ ВИРУС КРАСНУХИ, В РАЗЛИЧНЫХ ВОЗРАСТНЫХ ГРУППАХ

М. ТОТ

Автор с целью определения перенесенной краснухи в стране определял содержание нейтрализующих антител в 1937 сывороточных пробах, взятых от 0—80-летних лиц, в перевиваемой клеточной культуре РК—13 на основе подавления ЦПЭ.

Исследуемые сыворотки были подразделены на две группы. 1388 сывороточных проб, относящихся в первую группу, представляли собой материал, предназначенный для целей рутинного лабораторного исследования. Частота встречаемости нейтрализующих антител повышалась параллельно с возрастом. Первое резкое повышение выявилось в группе 16—20-летних, в которой частота встречаемости антител оказалась равной 74%. В группе 21—30-летних пропорция располагающих антителами достигла 85%. В возрасте между 31 и 70 годами серопозитивность колебалась практически на одинаковом уровне (88—81%).

Во вторую группу относились пробы крови 549 здоровых беременных женщин. Среди них 16,5% оказались негативными.

IN VITRO SYNTHESIS OF ANTIBODY TO PHAGES OF MYCOBACTERIUM PHLEI

L. HETÉY, E. VANDRA, I. FÖLDES

ОБРАЗОВАНИЕ IN VITRO АНТИТЕЛ ПРОТИВ ФАГОВ MYCOBACTERIUM PHLEI

Л. ХЕТЕИ, Е. ВАНДРА, И. ФЁЛДЕШ

Авторы изучали in vitro синтез антител против фагов *Mycobacterium phlei*. Суспензия из клеток селезенки кроликов, иммунизированных фагами *Mycobacterium phlei*, образовывала in vitro в присутствии фагов противофаговые антитела. Это явление частично подавлялось актиномицином Д.

EFFICIENCY OF SMALLPOX REVACCINATIONS REPEATED AT THREE YEAR INTERVALS. I. FOLLOW-UP OF THE IMMUNOLOGICAL STATUS OF THE STAFF OF AN INFECTIOUS DISEASE HOSPITAL

G. NYERGES, I. HOLLÓS, G. LOSONCZY

РЕЗУЛЬТАТЫ РЕВАКЦИНАЦИИ ПРОТИВ ОСПЫ, ПОВТОРЕННОЙ СПУСТЯ 3 ГОДА. I. ФОРМИРОВАНИЕ ИММУНОЛОГИЧЕСКОГО СТАТУСА У СОТРУДНИКОВ ИНФЕКЦИОННОЙ БОЛЬНИЦЫ

Г. НЬЕРГЕШ, И. ХОЛЛОШ, Г. ЛОШОНЦИ

Авторы изучали иммунитет у работников здравоохранения, успешно ревакцинированных 3 годами ранее, и его изменения, следовавшие за новой прививкой.

Спустя 3 года после успешной прививки среди обследованных нейтрализующие антитела не были обнаружены у 12%, а подавляющие гемагглютинацию антитела — у 32,8%. У привитых потенциальным прививочным материалом в 47,6% случаев наблюдали „major reaction”. Было замечено, что 23,8% equivocal-реакций протекает с образованием струпа, 28,6% — без струпа.

В большинстве случаев вслед за „major reaction” наблюдалось повышение титра подавляющих гемагглютинацию антител, в то время как в случае equivocal-реакций это явление наблюдалось только как исключение. В противоположность этому значительное размножение нейтрализующих антител происходило не только при „major”, но и при equivocal-реакциях, протекающих с образованием струпа. Поэтому представляется обоснованным equivocal форму реакций подразделить на две группы: „Equivocal₁ — выздоровление протекает с образованием струпа, Equivocal₂ — выздоровление без образования струпа.

Из-за отмеченного серологического расхождения реакцию equivocal₁ рассматривают как показатель сомнительной, а equivocal₂ — безрезультатной ревакцинации.

Изучение результатов ревакцинаций показало, что последние в 47% обследованных случаев в течение недели вызывают нарастание нейтрализующих антител. Таким образом, на основе серологических данных видно, что на быстрое защитное действие ревакцинаций можно рассчитывать всего лишь в половине случаев.

COMPARISON OF INTERFERON PRODUCTION IN VITRO BY LEUKOCYTES FROM HEALTHY AND POLYCYTHAEMIC PERSONS

GY. HADHÁZY, L. GERGELY, GY. NAGY, F. D. TÓTH

СРАВНЕНИЕ СПОСОБНОСТИ ЛЕЙКОЦИТОВ ЗДОРОВЫХ И БОЛЬНЫХ ПОЛИЦИТЕМИЕЙ ЛИЦ ВЫРАБАТЫВАТЬ ИНТЕРФЕРОН IN VITRO

ДЬ. ХАДХАЗИ, Л. ГЕРГЕЙ, ДЬ. НАДЬ, Ф. Д. ТОТ

Было проведено сравнение способности белых кровяных телец, происходящих от здоровых и больных полицитемией лиц, вырабатывать интерферон *in vitro*. В качестве индуктора использовали вирус парагриппа типа 1 (Sendai) и вирус Newcastle disease; определение интерферона проводили на перманентной культуре амниотических клеток человека с применением вируса везикулярного стоматита. Нашли, что способность лейкоцитов больных, страдающих polycythaemia vera, вырабатывать интерферон понижена по сравнению с таковой клеток здоровых доноров. На основе результатов всех проведенных экспериментов оказалось, что среднее значение титра интерферона в случае лейкоцитов больных полицитемией — 50, у здоровых — 220. По сравнению со средним контрольным титром среди 21 обследованного больного у 18 (85%) лейкоциты вырабатывали существенно меньше интерферона, у 3 (15%) — одинаковое количество. Авторы обсуждают результаты, сравнивая их с повышенной способностью лейкоцитов больных миелоидной лейкемией вырабатывать интерферон.

EXAMINATION IN VITRO OF THE VIRUS INHIBITORY ACTION OF A NEW BENZTHIAZOLE DERIVATIVE

L. GERCELY, F. D. TÓTH, GY. HADNÁZY

ИЗУЧЕНИЕ IN VITRO ПОДАВЛЯЮЩЕГО ДЕЙСТВИЯ НОВОГО ПРОИЗВОДНОГО BENZTHIAZOL-A НА ВИРУСНОЕ РАЗМНОЖЕНИЕ

Л. ГЕРГЕЙ, Ф. Д. ТОТ, ДЬ. ХАДХАЗИ

Авторы изучали подавляющее влияние нового производного benzthiazol-a (2[H'-pyridyl] benzthiazolmonomethyl-jodid) на размножение различных миксовирусных штаммов. Установили, что это химическое соединение селективно подавляет размножение штамма вируса гриппа А-2 (Singapore 4/57) на переживающей хорио-аллантоидной мембране. Прямым вируцидным действием не обладает, вирусную адсорбцию не подавляет, влияет на раннюю стадию размножения вируса внутри клетки.

FURTHER STUDIES ON THE LOCALIZATION AND IMMUNOLOGICAL PROPERTIES OF ANTIGENS INDUCED BY HERPES SIMPLEX VIRUS IN HEp-2 CELLS

L. GÉDER, F. LEHEL, E. JENEY, É. GÖNCZÖL

ДАЛЬНЕЙШЕЕ ИЗУЧЕНИЕ ЛОКАЛИЗАЦИИ И ИММУНОЛОГИЧЕСКИХ СВОЙСТВ АНТИГЕНОВ, ИНДУЦИРОВАННЫХ ВИРУСОМ HERPES SIMPLEX В КЛЕТКАХ HEp-2

Л. ГЕДЕР, Ф. ЛЕХЕЛ, Е. ЙЕНЕИ, Е. ГЕНЦЁЛ

Спустя 3—4 1/2 часа после заражения вирусом *Herpes simplex* в клетках HEp-2 наблюдали образование цитоплазматического, перинуклеарного, диффузного интрануклеарного и крупногранулярного (вдоль ядерной оболочки) антигена с помощью непрямой иммунофлуоресцентной техники, если препараты окрашивались сывороткой морских свинок, иммунизированных экстрактом из клеточной культуры, зараженной вирусом *Herpes simplex*. Спустя 4 1/2 — 5 часов после заражения появлялся интрануклеарный антиген, подобный включению. С той же сывороткой при обработке цитозин-арабинозидом в клетках могли быть выявлены только перинуклеарные и мелко рассеянные интрануклеарные иммунофлуоресцентные образования.

Методом агар-гелевой иммунной диффузии спустя 4 1/2 часа после заражения удалось отделить из клеточного экстракта 3 антигенных компонента, в то время как 6 компонентов могли быть отделены, если экстракт приготавливался спустя 16 часов после заражения.

Из пяти сывороток, используемых в исследованиях с применением иммунофлуоресцентной техники, 3 реагировали со всеми антигенами, тогда как 1 не реагировала с интрануклеарным антигеном, подобном включению, а другая реагировала только с теми антигенами, которые размещались в цитоплазме.

PLAQUE FORMATION BY DIFFERENT MEASLES VIRUS STRAINS IN A MONKEY KIDNEY CELL LINE

É. CSONKA, S. LETENAY

ОБРАЗОВАНИЕ БЛЯШЕК РАЗЛИЧНЫМИ ШТАММАМИ ВИРУСА КОРИ В КЛЕТОЧНОЙ ЛИНИИ ПОЧКИ ОБЕЗЬЯНЫ

Е. ЧОНКА, Ш. ЛЕТЕНАИ

Авторы изучали образование бляшек различными штаммами вируса кори в 3 различных клеточных культурах (первичная культура почки обезьяны, HeLa и клеточная линия почки обезьяны III/1 по Ruzieska). Наиболее характерные бляшки образовывались в клетках III/1.

Была проведена параллельная титрация в клеточных культурах III/1 и HeLa классическим методом и методом бляшек. Результаты совпадали.

Сравнили бляшковую морфологию 5 различных штаммов вируса кори в клетках III/1. Каждый из штаммов образовывал как малые, так и большие бляшки, и пропорция двух видов бляшек оказалась характерной для штаммов.

По мнению авторов, само по себе преобладание бляшек малых размеров не является показателем аттенуации данного штамма.

LYTIC FACTOR AND COMPETENCE IN *BACILLUS SUBTILIS*

S. HORVÁTH

ЛИТИЧЕСКИЙ ФАКТОР И КОМПЕТЕНЦИЯ У *BACILLUS SUBTILIS*.

Ш. ХОРВАТ

Клетки *B. subtilis* быстро аутолизуются, если хорошо функционирующие клетки попадают на обедненную питательную среду. Хорошо трансформируемые штаммы проявляют высокую аутолитическую активность. Активность литического фактора изменяется в течение культивирования.

Наивысшая точка компетенции наблюдается после 45—90 минут (в зависимости от применяемого штамма), когда литический фактор проявляет наибольшую активность. Развитие компетенции протекало быстрее, чем подъем литического фактора.

Фильтрат компетентных клеточных экстрактов и компетентных клеточных культур, а также лизозим не способствуют развитию компетенции. Не было найдено ни прямых, ни косвенных доказательств в том отношении, что литический фактор способствовал бы развитию компетенции.

INCIDENCE OF TRANSMISSIBLE DRUG RESISTANCE IN *PROTEUS* STRAINS

A. DOBOZY, H. HAMMER

ТРАНСМИССИВНАЯ РЕЗИСТЕНЦИЯ К АНТИБИОТИКАМ У ШТАММОВ *PROTEUS*

А. ДОБОЗИ, Х. ХАММЕР

Из 124 штаммов *proteus*, исследованных на чувствительность к антибиотикам, 22,6% оказались полирезистентными. 26 штаммов явились носителями R-фактора. Был найден двоякого построения R-фактор: один обуславливал резистенцию против сульфаниламида, стрептомицина, хлорамфеникола и тетрациклина, другой не содержал locus резистенции к тетрациклину.

При исследовании стабильности R-фактора выявили, что среди 3 штаммов из 2 сегрегируют тетрациклин-чувствительные клоны; это в одном случае сопровождалось и потерей резистенции к хлорамфениколу. Из 503 колоний третьего штамма сегрегант, чувствительный к упомянутым антибиотикам, не был обнаружен.

Величина стрептомициновой резистенции, происходящей от R-фактора, не превышала в материалах авторов концентрацию 100 μ г/мл.

EFFICACY OF SMALLPOX REVACCINATION REPEATED AT THREE-YEAR INTERVALS. II. COMPARATIVE STUDY OF THE INTERNATIONAL REFERENCE STRAIN (ELSTREE) AND THE HUNGARIAN VACCINE STRAIN (BUDAPEST)

G. NYERGES, I. HOLLÓS, G. LOSONCZY

ЭФФЕКТИВНОСТЬ РЕВАКЦИНАЦИИ ПРОТИВ ОСПЫ, ПОВТОРЕННОЙ СПУСТЯ ТРИ ГОДА. II. СРАВНИТЕЛЬНОЕ ИЗУЧЕНИЕ ВАКЦИН, ПРИГОТОВЛЕННЫХ ИЗ МЕЖДУНАРОДНОГО REFERENCE-ШТАММА (ELSTREE) И ВЕНГЕРСКОГО ШТАММА (BUDAPEST).

Г. НЬЕРГЕШ, И. ХОЛЛОШ, Г. ЛОШОНЦИ

Авторы проводили ревакцинацию взрослых, эффективно привитых 3 годами ранее, дермовакцинами, приготовленными из международного reference-штамма и штамма „Budapest”. Количество вируса (БОЕ) в обеих вакцинах было тождественным.

Изучали, как сказывается на результате прививки изменение количества вируса, вносимого прививкой. При введении относительно небольшого количества вакцины пропорция major-реакций была вдвое больше среди привитых вакциной „Budapest”, чем среди привитых reference-вакциной. Если количество вводимой вакцины в случае обоих прививочных материалов равномерно увеличивали, то пропорция major-реакций среди привитых reference-вакциной значительно возрастала, в то время как среди вакцинированных вакциной „Budapest” она практически оставалась без изменений.

Средние титры нейтрализации привитых двумя видами вакцин с одинаковым результатом по существу не расходились между собой. Титры в РИГА тоже обычно совпадали, исключая средний титр, определенный спустя три недели после прививки у успешно привитых.

Была проведена математическая оценка результата прививок у молодых (20—40-летних) и пожилых (41—60-летних) лиц. В группе пожилых значительно больше было сомнительных результатов и меньше была пропорция безуспешно привитых, чем в группе молодых. Исходя из серологических изменений, следующих за прививками, между группой молодых и группой пожилых не было найдено никакой разницы.

COMPARATIVE LABORATORY STUDY OF VACCINES PREPARED FROM THE INTERNATIONAL REFERENCE STRAIN AND FROM THE „BUDAPEST” STRAIN OF VACCINIA VIRUS

G. NYERGES, J. SZATHMÁRY, A. BENKŐ

СРАВНИТЕЛЬНОЕ ЛАБОРАТОРНОЕ ИССЛЕДОВАНИЕ ВАКЦИН, ПРИГОТОВЛЕННЫХ ИЗ МЕЖДУНАРОДНОГО REFERENCE-ШТАММА ВАКЦИНИИ И ШТАММА „BUDAPEST”

Г. НЬЕРГЕШ, Й. САТМАРИ, А. БЕНКЁ

Авторы методом скарификации и внутрикожного введения изучали на кроликах титры яичной и телачьей дермовакцин с одинаковой концентрацией БОЕ, приготовленных одинаковым способом из штамма „Budapest”, используемого в Венгрии в производстве прививочного материала против оспы, и reference-штамма (Elstree). Кроме того, определили титры $L_{D_{50}}$ на мышях, куриных эмбрионах и $CPID_{50}$ на культуре почки обезьяны.

Титр $L_{D_{50}}$ вакцины на мышях, приготовленной из штамма „Budapest”, был умеренно, а на куриных эмбрионах — определенно выше, чем таковой вакцины с подобной концентрацией БОЕ из reference-штамма.

Штамм „Budapest” не считают нейровирулентным штаммом вакцинии, так как вызываемая им морфология лезий не отличается от таковой reference-штамма, и его вирулентность в мышях не усиливается при интрацеребральных пассажах.

Расходящиеся лабораторные особенности вакцин, приготовленных из двух штаммов, сказываются и на различии вакцинной take-пропорции у привитых ими лиц.

THE LIPID COMPOSITION OF PATHOGENIC AND SAPROPHYTIC MYCOBACTERIA

S. TUBOLY

ИССЛЕДОВАНИЕ ЛИПОИДНЫХ ВЕЩЕСТВ У ПАТОГЕННЫХ И САПРОФИТНЫХ МИКОБАКТЕРИЙ

Ш. ТУБОЙ

Автор исследовал липоидные вещества патогенных и сапрофитных штаммов микобактерий. Методом тонкослойной хроматографии в липоидных экстрактах штаммов *Mycobacterium tuberculosis* и *Mycobacterium bovis* удалось определить одинаковое число фракций с одинаковым значением Rf. Штаммы *Mycobacterium avium* тем отличаются от указанных выше штаммов, что между восковыми веществами и триглицеридами отделяется ещё одна фракция. Свободные жирные кислоты сапрофитных штаммов выявить не удалось.

Жирные кислоты с C 12 — C 22 в штаммах *Mycobacterium tuberculosis* и *Mycobacterium bovis* были определены анализом газовой хроматографии. Всё расхождение между ними заключалось в том, что в штаммах *Mycobacterium bovis* встречалась также и жирная кислота с C 18 : 2. Липоидные вещества штаммов BCG и AN₅ не содержат ненасыщенных жирных кислот. В штаммах *Mycobacterium avium* встречаются жирные кислоты с содержанием от 12 до 18 углеродных атомов; в последнем случае наряду с C 18 : 1 и C 18 : 2 встречался и C 18 : 3.

Сапрофитные штаммы оказались более бедными в отношении ненасыщенных жирных кислот.

Липоид-гаптены, выявленные из штаммов *Mycobacterium tuberculosis*, *Mycobacterium bovis* и *Mycobacterium avium*, не оказались видоспецифическими, так как могли быть выявлены не только в гомологичных системах, но — за исключением иммунных сывороток сапрофитных микобактерий — и в перекрестных реакциях.

INVESTIGATIONS INTO THE TESTING OF METISAZONE PREPARATIONS

I. HOLLÓS, G. NYERGES

НАБЛЮДЕНИЯ ОТНОСИТЕЛЬНО ИССЛЕДОВАНИЙ ПРЕПАРАТОВ METISAZON-a.

И. ХОЛЛОШ, Г. НЬЕРГЕШ

Измеряли токсичность двух препаратов metisazon-a на однослойной культуре фибробластов куриного эмбриона, 12-дневном курином эмбрионе и мышах, привитых внутрибрюшинно. На мышах наблюдали как токсический симптом выделение кровяного стула, нарушение дефекации, а также окрашивание мочи в желтый цвет.

Для исследований на эффективность хорио-аллантоисная оболочка куриного эмбриона оказалась более пригодной, чем клеточная культура фибробластов куриного эмбриона.

На мышах было проведено изучение трех доз обоих препаратов metisazon-a против двух доз ненейровирулентного штамма вакцины „Budapest”. Об эффективности судили по подавлению вирусного размножения, наблюдаемого в мозгу мышей. Этот метод довольно точен, но очень трудоёмок.

Соотношение между наибольшей, но ещё не токсической дозой и наименьшей дозой, ещё оказывающей антивирусное действие, на культуре фибробластов куриного эмбриона было 1 : 1 — 4 : 1, на хорио-аллантоисной мембране — 6 : 1 — 12 : 1, на мышах — 16 : 1.

Представляется необходимой стандартизация методов изучения эффекта анти-вирусных средств. В случае вируса вакцины подающимся стандартизации является исследование на хорио-аллантоисной мембране.

Оба исследованных препарата metisazon-a по эффективности и токсичности оказались одинаковыми.

EFFECT OF FLAVONIDS AND RELATED SUBSTANCES. II. ANTIVIRAL EFFECT OF QUERCETIN, DIHYDROQUERCETIN AND DIHYDROFisetin

M. BAKAY, I. MUCSI, I. BÉLÁDI, M. GÁBOR

ИЗУЧЕНИЕ ДЕЙСТВИЯ ФЛАВОНОИДОВ И РОДСТВЕННЫХ СРЕДСТВ. II. АНТИВИРАЛЬНОЕ ДЕЙСТВИЕ QUERCETIN-a, DIHYDROQUERCETIN-a и DIHYDROFisetin-a.

М. БАКАИ, И. МУЧИ, И. БЕЛАДИ, М. ГАБОР

Изучали действие quercetina, dihydroquercetin-a и dihydrofisetin-a, оказываемое на вирусы. Нашли, что все три флавоноида располагают вируцидной активностью против *Herpesvirus hominis* и *Herpesvirus suis*. Quercetin и dihydroquercetin инактивировали вирусы герпеса примерно в одинаковой степени. Вирус парагриппа 3 типа проявлял чувствительность только к quercetin-y, в то же время вирус полиомиелита 2 типа оказался резистентным к исследуемым флавоноидам.

В наших опытах quercetin и dihydroquercetin обладали не только вируцидным, но и вирусостатическим действием, так как их введение даже после вирусного заражения понижало инфективный титр *Herpesvirus hominis*.

THE EFFECT OF HYPOTHERMIA ON THROMBOHAEMORRHAGIC REACTIONS

T. SZILÁGYI, S. TÓTH, G. LÉVAI, L. KASSAY

ВЛИЯНИЕ ГИПОТЕРМИИ НА ТРОМБОГЕМОРРАГИЧЕСКУЮ РЕАКЦИЮ

Т. СИЛАДЬИ, Ш. ТОТ, Г. ЛЕВАИ, Л. КАШШАИ

В поджелудочную и слюнные железы кроликов впрыскивали эндотоксин, затем вызывали реакцию Schwartzman-a. Поскольку действующую дозу вводили внутривенно охлажденным животным, реакция отсутствовала или была очень слабой. Охлаждением животных можно было подавить и реакцию Schwartzman-a, вызванную гомогенизатом лейкоцитов. В противоположность этому некротическую реакцию кожи, подготовленную SeCl_4 +адреналин, гистамином или 5—HF и вызванную NaCl, нельзя было подавить понижением температуры тела.

CONVERSION OF STEROIDS BY ALCALIGENES FAECALIS

G. WIX, K. ALBRECHT, G. AMBRUS, A. SZABÓ

ПРЕОБРАЗОВАНИЕ СТЕРОИДОВ ПОД ВЛИЯНИЕМ ALCALIGENES FAECALIS

Г. ВИКС, К. АЛБРЕХТ, Г. АМБРУШ, А. САБО

Среди изолятов, разлагающих желчную кислоту, идентифицировали один микроорганизм как *Alcaligenes faecalis*. Установили, что микроорганизм располагает $\Delta 1$ и $\Delta 4$ дегидрогеназой, 3β -гидроксидегидрогеназой, $\Delta 5$ -, $\Delta 4$ изомеразой и эстеразной активностью; разрывает структуру стерана между 9 и 10 атомами углерода. Сравнили скорость метаболизации соединений андростановой и прегнановой структуры.

INCIDENCE AND BIOLOGICAL PROPERTIES OF METHICILLIN AND OXACILLIN RESISTANT STAPHYLOCOCCUS AUREUS STRAINS

F. ROZGONYI, M. ILLÉS, I. RÉDAI

ЧАСТОТА ВСТРЕЧАЕМОСТИ И БИОЛОГИЧЕСКИЕ СВОЙСТВА ШТАММОВ STAPHYLOCOCCUS AUREUS, РЕЗИСТЕНТНЫХ К МЕТИЦИЛЛИНУ И ОКСАЦИЛЛИНУ

Ф. РОЗГОНЬИ, М. ИЛЛЕШ, И. РЕДАИ

Частота встречаемости штаммов *Staphylococcus aureus*, резистентных к метициллину и оксациллину, повышается. В клиниках Медицинского института г. Дебрецен такие штаммы были выделены в 1966 г. с частотой 4,6% и 4,3%, в 1967 г. — 7,9% и 5,7%.

Свойства *in vitro*, определяющие патогенность штаммов, резистентных и чувствительных к метициллину и оксациллину, не показывали значительного расхождения.

Резистентные штаммы встречаются в каждой фаговой группе, но среди них преобладают представители фаговой группы III и нетипизирующиеся.

Штаммы с естественной резистентностью к метициллину и оксациллину состоят из смешанной популяции; встречаемость резистентных единиц, образующих колонии, — 1/23 000 — 79 000.

Имеется характерная разница между типом размножения, происходящего в метициллине, у штаммов с естественной и искусственной резистентностью к метициллину.

RELATIONSHIP BETWEEN RESISTANCE AND INACTIVATION OF METHICILLIN AND OXACILLIN IN STAPHYLOCOCCUS AUREUS

F. ROZGONYI, I. RÉDEI

ИЗУЧЕНИЕ СВЯЗИ МЕЖДУ ИНАКТИВАЦИЕЙ МЕТИЦИЛЛИНА И ОКСАЦИЛЛИНА И РЕЗИСТЕНЦИЕЙ В СЛУЧАЕ ШТАММОВ STAPHYLOCOCCUS [AUREUS]

Ф. РОЗГОНЬИ, И. РЕДАИ

Метициллин и оксациллин инактивируются штаммами *Staphylococcus aureus*, предварительно не индуцированными метициллином или оксациллином, состоящими из гетерогенной популяции, резистентными к метициллину-оксациллину и продуцирующими пенициллиназу.

Инаktivация метициллина начинается в стационарной фазе размножения и усиливается вместе с продвижением последней; это противоположно инаktivации оксациллина, которая начинается в логарифмической стадии.

Для размножения штаммов *Staphylococcus aureus*, естественно резистентных к метициллину и оксациллину, нет необходимости в инаktivации антибиотиков. Инаktivация метициллина и оксациллина — вторичное явление и не является причиной резистентности.

REGULATION OF ALKALINE PHOSPHATASE PRODUCTION IN THE AUXOTROPHS OF *BACILLUS ANTHRACIS*

A. DOBOZY, E. MARJAI, G. IVÁNOVICS

РЕГУЛЯЦИЯ ПРОДУКЦИИ ЩЕЛОЧНОЙ ФОСФАТАЗЫ В АУКСОТРОФНЫХ ШТАММАХ *BACILLUS ANTHRACIS*

А. ДОБОЗИ, Е. МАРЬЯИ, Г. ИВАНОВИЧ

Репрессия продукции щелочной фосфатазы в прототрофных и ауксотрофных штаммах *Bacillus anthracis* протекает по трем типам. Для I типа характерна репрессия, постепенно возникающая вместе с ростом концентрации ортофосфата. Так ведут себя прототрофные штаммы, а также пиримидин- и витамин-зависимые ауксотрофы. К этому типу относятся ещё те пурин-ауксотрофы, в которых дефект находится перед замыканием пуринового кольца. Специфически аденин-зависимые ауксотрофы, в которых отсутствует энзимная активность (*adenylosuccino synthase* или *adenylosuccino lyase*), представляют репрессию второго типа. В этом случае репрессия протекает в двух стадиях. Что касается их фенотипов, то 4 из 5 гуанин-зависимых штаммов представляют третий тип репрессии, при которой уже самая минимальная концентрация фосфата та, которая позволяет только субоптимальный рост бактерий, обеспечивает полную репрессию продукции щелочной фосфатазы. Происходящее превращение ауксотрофов в прототрофы восстанавливает тип репрессии, характерный для первоначального, родительского штамма. Как видно, регуляция щелочной фосфатазы находится в связи с биохимическим путем синтеза пурина, то есть регулирование продукции этого катаболического экзосензима зависит от некоторых метаболических фаз синтеза пурина.

THE ULTRASTRUCTURE OF THE VACCINIA VIRUS GROWING IN THE EMBRYONATED EGG

I. HOLLÓS, B. LOVAS

ЭЛЕКТРОННО-МИКРОСКОПИЧЕСКОЕ ИЗУЧЕНИЕ РАЗМНОЖЕНИЯ ВИРУСА ВАКЦИНИИ В ЭМБРИОНИРОВАННОМ ЯЙЦЕ

И. ХОЛЛОШ, Б. ЛОВАШ

С помощью электронного микроскопа изучали размножение вируса вакцинии в клетках хорио-аллантоидной мембраны эмбрионированного куриного яйца.

Нашли, что вирионы без ориентации адсорбируются к клеткам хориона и проникают в последние наподобие чужого тела. Разрушение наружной мембраны вирионов и высвобождение *virus-core* следует одновременно с эклипсом. Вслед за этим около клеточного ядра хорошо выявляемый характерный, вирусной природы матрикс (вирусная фабрика) и незрелые вирусные частицы. Вирусные частицы постепенно отодвигаются к периферическим частям цитоплазмы, и вскоре появляются переходные и зрелые вирусные частицы.

Для морфологических исследований авторы точно считают приемлемым эмбрионированное яйцо, так же как и клеточные культуры.

HALIDE AND ALKALI ION UPTAKE BY STREPTOMYCES AUREOFACIENS

Z. BÖSZÖRMÉNYI, E. CSEH, M. JÁRAI

ТРАНСПОРТ ГАЛОИДА И АЛКАЛЬНОГО ИОНА STREPTOMYCES AUREOFACIENS

З. БЁСЁРМЕНЬИ, Е. ЧЕХ, М. ЯРАИ

Провели сравнительное изучение транспорта галоида и алкального иона штаммов *Streptomyces aureofaciens* (B—28 и СДСД—314).

Приём $42K$ — посредственный процесс транспортировки, который всего за 40—60 минут доводит до уровня равновесия, существенно превышающего наружную концентрацию. Транспорт K подавляется азидом. Различие K/Na сильно способствует K .

Балансное содержание бромидов остается ниже наружной концентрации и является её линейным зависимым. Концентрация равновесия устанавливается меньше чем за 5 минут, и содержание бромидов в течение короткого времени может быть вымыто или заменено. Азид повышает балансное содержание бромидов. На основании данных считается вероятным, что бромид проникает в клетку с непосредственным процессом, и концентрация равновесия определяется распределением по Donnan-у. Повышение осмолярности растворов понижает как транспорт K , так и балансное содержание Na или бромидов. В случае *Streptomyces aureofaciens* не действует механизм по осмотической регуляции, основывающийся на транспорте K .

Особенности транспорта исследованных штаммов *Streptomyces* показывают меньшую количественную разницу (например, балансное содержание K и Na штамма СДСД—314 выше, содержание бромидов ниже, чем таковые линии B—28), однако потеря продукции хлортетрациклина у линии СДСД—314 не может быть приписана мутации транспорта галоида.

DEVELOPMENT OF IMMUNOLOGICAL MEMORY IN MICE TO PHOSPHORYLASE ANTIGEN. I. THE EFFECT OF DOSE AND INTERVAL OF ANTIGENIC STIMULI

I. JÓKAY, E. KARCZAG

ИЗУЧЕНИЕ РАЗВИТИЯ ИММУНОЛОГИЧЕСКОЙ ПАМЯТИ У МЫШЕЙ С ПОМОЩЬЮ ФОСФОРИЛАЗНОГО АНТИГЕНА

I. ВЛИЯНИЕ ДОЗЫ АНТИГЕНА И ИНТЕРВАЛА МЕЖДУ АНТИГЕННЫМИ СТИМУЛАМИ

И. ЙОКАЙ, Е. КАРЦАГ

С помощью фосфорилазного антигена изучали на мышах зависимость первичного и вторичного иммунного ответа от антигенной дозы и влияние интервала между двумя антигенными дозами, находящимися ниже порога, на уровень плазматических антител. Энзимнейтрализующие антитела сыворотки после первого и второго иммунного раздражения оказались типами 7S и резистентными к меркаптоэтанолу. На 0,01—1,0 мг антигена индуцируется чисто иммунологическая память без выявляемого образования антител, тогда как на 1—5 мг антигена появляется образование антител в небольшом количестве. 1 мг пороговой дозы первичного антигена (если время полураспада примем за 400 минут) за 40—60 часов понижается в организме до пороговых значений вторичного иммунного ответа (2—8 μg). Две антигенных дозы со значениями ниже порога были даны с интервалами в 1, 2, 3, 4, 5 дней; оптимальным оказался двухдневный интервал. Исходя из этих данных, авторы сделали вывод, что раннее формирование иммунологической памяти (X—Y трансформация) находится между 2—2,5 днями.

С различными интервалами давали две антигенных дозы со значениями ниже порога (700 μg и 250 μg) и изучали дальнейшее формирование иммунологической памяти. В случае 2—8-дневного интервала уровень антител был одинаковый по величине с максимальной первичной реакцией; при интервале 8—17 дней возникло экспоненциальное повышение, а после применения на 20—65 день вторичного антигенного раздражения формировался постоянный высокий уровень антител.

NEW OBSERVATIONS ON THE DIFFERENTIATION OF MYCOBACTERIUM TUBERCULOSIS AND MYCOBACTERIUM BOVIS

T. FODOR, E. VANDRA

НОВЫЕ ДАННЫЕ К ОТДЕЛЕНИЮ MYCOBACTERIUM TUBERCULOSIS И MYCOBACTERIUM BOVIS

Т. ФОДОР и Е. ВАНДРА

Из 104 штаммов, происходящих от больных туберкулёзом, характерную для *M. tuberculosis* реакцию в нитратной пробе удалось получить в 101 случае, в нитиновом тесте — в 95 случаях и при внутрикожном введении кроликам — в 103 случаях, тогда как при фаготипизации — только в 23 случаях. Таким образом, для дифференцировки более пригодными являются опыты на животных и биохимические реакции, чем фаготипизация.

THE EFFECT OF RAPHANIN ON THE MULTIPLICATION OF SOME RNA AND DNA VIRUSES IN AND INTERFERON PRODUCTION BY, CULTURED CELLS

I. ROSZTÓCZY

ДЕЙСТВИЕ РАФАНИНА НА РАЗМНОЖЕНИЕ РНК- И ДНК-ВИРУСОВ, А ТАКЖЕ НА ПРОДУКЦИЮ ИНТЕРФЕРОНА КЛЕТКАМИ КУЛЬТУРЫ

И. РОСТОЦИ

В культуре фибробластов куриного эмбриона изучали влияние рафанина на размножение вирусов псевдобешенства, вакцинии, лесов Семлики, синдбис, везикулярного стоматита, Чукунгуния, а также на продукцию интерферона. Рафанин сильнее подавляет размножение ДНК-вирусов, чем РНК-вирусов. На основе действия, проявленного на продукцию интерферона, сделали заключение, что вирусоподавляющее влияние рафанина основывается на нарушении клеточного обмена веществ.

THE EFFECT OF SORBIC ACID ON THE GROWTH OF THE YEAST PROCANDIDA (CANDIDA) ALBICANS

T. DEÁK, E. K. NOVÁK

ДЕЙСТВИЕ СОРБИНОВОЙ КИСЛОТЫ НА РАЗМНОЖЕНИЕ PROCANDIDA (CANDIDA) ALBICANS

Т. ДЕАК, Е. К. НОВАК

Авторы изучали подавляющее влияние сорбита калия на размножение *Procandida albicans* в разных инкубационных системах. На основе постоянной скорости размножения, продолжительности lag-фазы и измерения окончательной клеточной плотности установили, что среди анаэробных условий подавляющее действие сорбита калия проявляется сильнее, чем в аэробных условиях независимо от состава питательной среды. Причину подавления меньшей степени, наблюдаемого в аэробных условиях, авторы приписывают тому, что при аэробных условиях культуры детоксицируют сорбит. Степень детоксикации зависит от состава питательной среды. Цистеин и глутатион проявляют как раз противоположное действие, чем это можно было ожидать судя по литературным данным. Вместо того, чтобы помешать подавляющему действию сорбита, они усиливали это влияние.

STRESS REACTION OF MICE TREATED WITH ANTILYMPHOCYTE SERUM

P. ANDERLIK, S. BÁNOS, H. SZERI

РЕАКЦИЯ МЫШЕЙ, ПОЛУЧАВШИХ АНТИЛИМФОЦИТНУЮ СЫВОРОТКУ, НА ДЕЙСТВИЕ ШТРЕССА

П. АНДЕРЛИК, Ш. БАНОШ, Х. СЕРИ

В мышцах с лимфопенией, вызванной антилимфоцитной сывороткой, лимфопеническая реакция на действие стресса отсутствовала или понижалась. Размеры лимфопенической реакции показывали взаимосвязь с абсолютными числами лимфоцитов. При очень низких числах лимфоцитов наблюдали повышенную чувствительность к холоду.

THE EFFECT OF GOLD SALT TREATMENT ON LOCAL SHWARTZMAN PHENOMENON

T. SZILÁGYI, S. TÓTH, L. MUSZBEK, G. LÉVAI, J. LACZKÓ

ДЕЙСТВИЕ СОЛИ ЗОЛОТА НА МЕСТНЫЙ ФЕНОМЕН ШВАРЦМАНА

Т. СИЛАДЬИ, Ш. ТОТ, Л. МУСБЕК, Г. ЛЕВАИ, Й. ЛАЦКО

На месте кожной реакции, вызванной подготовительной дозой эндотоксина, вводили один и два раза препарат соли золота. Установили, что обработка солью золота в большей степени понижает и совершенно подавляет развитие явления Шварцмана. Предполагают, что соединение золота, примененное местно, оказывает эффект на лизосомы клеток и их ферменты.

BIOCHEMICAL AND CULTURAL CHARACTERS OF SEROLOGICALLY GROUPEP PSEUDOMONAS AERUGINOSA STRAINS

B. LÁNYI

БИОХИМИЧЕСКИЕ И КУЛЬТУРАЛЬНЫЕ СВОЙСТВА СЕРОГРУПП PSEUDOMONAS AERUGINOSA

Б. ЛАНЫ

Автор изучал свойства 233 штаммов, относящихся в различные серогруппы *Pseudomonas aeruginosa*, отобранных по частоте встречаемости серологических подгрупп и происходящих из разных источников.

На основе полученных результатов и данных других авторов свойства *Pseudomonas aeruginosa* могут быть охарактеризованы следующим образом: Кислотообразование на среде, бедной источником органического азота. 100%-но отрицательна в отношении адонита, дульцита, М-инозита, инулина, лактоза-д-мальтозы, раффинозы, L-рамнозы, салицина, д-сорбита, сахарозы; положительна в отношении Д-арабинозы — 99,1%, L-арабинозы — 98,4%, Д-галактозы — 97,4%, Д-глюкозы (оксидативно) — 98,6%, Д-маннозы — 99,5%, Д-ксилозы — 98,5%. Только часть штаммов разлагала Д-трехалозу и L-ксилозу; так как 24,4 и 24% штаммов после повторных исследований привели к расходящимся результатам, эти две реакции не могут быть использованы для отделения биотипов. У большинства штаммов только более чувствительными методами можно выявить небольшое количество кислоты, вырабатываемой из глицерина, Д-левулозы и Д-маннита.

Использование как источника углерода. 100%-но отрицательна в отношении адонита, Д-арабинозы, L-арабинозы, дульцита, Д-галактозы, М-инозита, инулина, лактозы, Д-мальтозы, Д-маннозы, раффинозы, L-рамнозы, салицина, Д-сорбита, сахарозы, Д-ксилозы, L-ксилозы, Д-тартарата; положительна в отношении этанола — 99,7%, глицерина — 99,7%, глюкозы — 99,7%, D-левулозы — 99,5%, Д-маннита — 98,7%, бензоата — 99,7%, цитрата — 99,9%, лактата — 99,8%, ацетата — 99,8%, малоната 99,7%, пропионата — 99,6%, сукцината — 99,7%. Непостоянные результаты были получены в случае трехалозы; в метаноле штаммы обычно проявляли позднее и слабое размножение. *Прочие реакции углеводородного обмена веществ.* Проба метилового красного, *Voges-Proskauer-a*, эскулина и гидролиза крахмала 100%-но отрицательна. *Вещества, содержащие нитроген.* Аргинин дигидролаза — позитивно на 100%, лизин и орнитин декарбоксилаза, триптофанаминидаза — отрицательно на 100%. Расплавление желатина — 99,7%, липолитическая активность — 99,3%, нитратная редукция — 100%, использование уреума как источника N — положительно на 99,7%; продукция индола — отрицательно на 100%. *Прочие свойства.* Продукция сероводорода из тиосульфата — отрицательно на 100%, проба на каталазу и оксидазу, продукция гемолизина положительна на 100%, флуоресцина — на 98,8%, пиоцианина — на 86,4%; движение — положительно на 99,5%, рост при 41–42° C — 99,9%, рост при 4–5° C — 0,2%. Все штаммы размножались в присутствии бриллиантового зеленого, цетилпиридин-бромид, калий-цианида, мертиола, хлористого натрия, натрий-тауроколата и хлористого трифенилтетразолия в концентрациях, подавляющих большинство других бактерий, а также на агаре, содержащем бриллиантовый зеленый, дезоксихолат-цитрат, бисмут-сульфат и эозинметиленовый синий.

В соответствии с данными, *Pseudomona aeruginosa* является гомогенным видом, внутри серологических подгрупп которого отделение биотипов практически едва возможно.

MAINTENANCE OF TOXOPLASMA GONDII IN PRIMARY HUMAN AMNIOTIC CELL CULTURE

G. CSÓKA, G. KULCSÁR

ПОДДЕРЖАНИЕ TOXOPLASMA GONDII В ПЕРВИЧНОЙ КУЛЬТУРЕ АМНИОТИЧЕСКИХ КЛЕТОК ЧЕЛОВЕКА

P. ЧОКА, Г. КУЛЧАР

Был разработан простой метод для непрерывного поддержания *T. gondii*. В течение 8 месяцев провели 45 пассажей, при этом не было замечено никаких изменений ни в микроскопической морфологии паразитов, ни в их способности размножаться, ни в их патогенности для животных. Методом трипсинизации можно было освободить из массы погибших клеток всё количество токсоплазм.

VIRULENCE OF SHIGELLA STRAINS ISOLATED FROM DYSENTERIC PATIENTS AND SYMPTOMLESS CARRIERS

L. KEREKES

ВИРУЛЕНТНОСТЬ ШТАММОВ SHIGELLA, ВЫДЕЛЕННЫХ ОТ БОЛЬНЫХ ДИЗЕНТЕРИЕЙ И КЛИНИЧЕСКИ ЗДОРОВЫХ БАЦИЛЛОНОСИТЕЛЕЙ

Л. КЕРЕКЕШ

Автор на основе конъюнктивального заражения морских свинок сравнивал вирулентность штаммов *Shigella*, выделенных от больных и бациллоносителей. В вирулентности штаммов не было найдено количественной разницы. Значение ИД₅₀ штамма 3а *Shigella flexneri* было выше, чем среднее значение ИД₅₀ штаммов *Shigella flexneri*. В случае штаммов *Shigella sonnei* среднее значение ИД₅₀ было на 1 log выше, чем в случае штаммов *Sh. flexneri* независимо от того, происходили ли эти штаммы от клинически здоровых бациллоносителей или от больных. На основе данных опыта клинически здоровых бациллоносителей надо рассматривать как потенциально полноценный источник инфекции.

REGULATION OF ALKALINE PHOSPHATASE SYNTHESIS IN AUXOTROPHIC MUTANTS OF BACILLUS SUBTILIS

A. DOBOZI, H. HAMMER

РЕГУЛЯЦИЯ СИНТЕЗА ЩЕЛОЧНОЙ ФОСФАТАЗЫ В АУКСОТРОФНЫХ МУТАНТАХ B. SUBTILIS

А. ДОБОЗИ, Х. ХАММЕР

Концентрация фосфата в питательной среде 0,46—0,66 мМ репрессирует продукцию щелочной фосфатазы штаммов *B. subtilis*. Исключение составляют аденин-зависимые штаммы, которые даже при самой низкой концентрации ионов фосфата, обеспечивающей рост, располагают только такой низкой фосфатазной активностью, как и дикие штаммы после полной репрессии.

Результаты опытов по трансформации указывают на то, что за пониженную фосфатазную активность ответственна мутация, возникшая в структурных генах энзимов биосинтеза аденина. В одинаковой степени пониженную фосфатазную активность показывают те штаммы, в которых мутация находится в структурном гене *adenylosuccino synthase* или *adenylosuccino lyase*.

В структурном гене *adenylosuccino synthase* может возникнуть и такая мутация, которая прекратит биосинтез аденина, но не оказывает влияния на активность фосфатазы.

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